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VAGINAL MORPHOLOGICAL CHANGES IN CERVICAL CANCER SURVIVORS

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To my family with love

ABSTRACT

Background: Cervical cancer is globally the fourth most common cancer in women and has generally a good prognosis. Cervical cancer survivors report persistent changes in their sexual function, which result in considerable distress. The majority of new cases are diagnosed in young or middle-aged women. Consequently, a rising number of women are at risk for chronic side effects of the treatment. The aim of this thesis was to investigate the morphology of the vaginal wall and the expression of sex steroid receptors in cervical cancer survivors treated with radiotherapy.

Methods: Via clinical examination the degree of vaginal atrophy, pelvic fibrosis and telangiectasia were estimated. We collected vaginal biopsies, which underwent morphometric analyses focused on elastin, collagen, epithelial structures, blood vessels and nerve fibers. Radiation dose at biopsy site was calculated and sex steroid hormone levels were analyzed. We also analyzed the expression and distribution of sex steroid hormone receptors by real-time PCR and immunohistochemistry (IHC). Additionally, a questionnaire designed to assess sexual function was filled out.

Results: The survivors had marked morphological vaginal changes, most prominent in the survivors that had received the highest radiation dose at the biopsy site. Clinical findings of atrophy, pelvic fibrosis and shortened vagina were present in most cancer survivors. Signs of elastosis with thick aggregated elastin fibers were found throughout the connective tissue. High-density collagen (fibrosis) in the connective tissue was more common among the survivors, most prominent in survivors that had received external radiation. The vaginal epithelium volume was reduced despite no difference in serum estradiol between cancer survivors and controls. In biopsies from the vaginal wall, lower expression of estrogen receptor alpha (ERα) at both mRNA and protein levels was found. In the survivors with high radiation dose at biopsy site, the immunostaining of ERα and androgen receptor (AR) was lower in the epithelium and the stroma, compared to survivors with minimal radiation dose. The cervical cancer survivors reported more physical sexual symptoms. The highest relative risk was found for insufficient vaginal lubrication, vaginal inelasticity, reduced genital swelling when sexually aroused and for reduction of vaginal length.

Conclusion: The vaginal wall is markedly changed in irradiated cervical cancer survivors. The connective tissue is remodeled with elastosis and radiotherapy-induced fibrosis, and the vaginal epithelium volume is reduced, compared to controls with similar levels of estradiol. Reduction in ERα and AR expression in the vaginal mucosa after radiotherapy may alter the responsiveness to hormonal treatment. The morphological changes in the vaginal wall correspond to the clinical findings of fibrosis and atrophy and can be one important explanation of the sexual dysfunction in the cervical cancer survivors.

LIST OF SCIENTIFIC PAPERS

I. Hofsjö A, Bohm-Starke N, Blomgren B, Jahren H, Steineck G, Bergmark K.
Radiotherapy-induced vaginal fibrosis in cervical cancer survivors.
Acta Oncol. 2017;56(5):661-666.

II. Hofsjö A, Bergmark K, Blomgren B, Jahren H, Bohm-Starke N.
Radiotherapy for cervical cancer – impact on the vaginal epithelium and sexual function.
Acta Oncol. 2017;15:1-88 doi: 10.1080/0284186X.2017.1400684.
[Epub ahead of print]

III. Hofsjö A, Bohm-Starke N, Bergmark K, Masironi B, Sahlin L.
Sex steroid hormone receptor expression in the vaginal wall in cervical cancer survivors after radiotherapy
In manuscript

CONTENTS

LIST OF ABBREVIATIONS

AR	androgen receptor
BLS	distance from basal layer to surface
BT	brachytherapy
CI	confidence interval
CT	computed tomography
CTCAE	common terminology criteria for adverse events
CTGF	connective tissue growth factor
DPD	interdermal papilla distance
DPS	distance from dermal papilla top to epithelial surface
DPW	dermal papilla width
E1	estrone
E2	estradiol
E3	estriol
EBRT	external beam radiation therapy
FIGO	Federation Internationale de Gynecologie et d'Obstetrique
FSH	follicle-stimulating hormone
GLOBOCAN	WHO global cancer database, www.globocan.iarc.fr
GPER	G-protein coupled estrogen receptor
HPV	human papilloma virus
HR-CTV	high-risk clinical target volume
HRT	hormone replacement therapy
IGABT	image guided adaptive brachytherapy
IHC	immunohistochemistry
IMRT	intensity modulated radiation therapy
IQR	interquartile range
LDR	low dose rate
LH	luteinizing hormone
LVSI	lymphovascular space involvement
MMP9	metalloproteinase 9
MMPs	matrix metalloproteinase
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid

NORDCAN	WHO Nordic cancer database, www-dep.iarc.fr
Pap	Papanicolaou
PCR	polymerase chain reaction
PDR	pulsed dose rate
PET	positron emission tomography
PGP 9.5	protein gene product 9.5
PR	progesterone receptor
RIA	radioimmunoassay
RPLP0	ribosomal protein P0
RR	relative risk
SHBG	sex steroid-hormone binding globulin
TNM	tumour, nodes, metastasis

1 INTRODUCTION

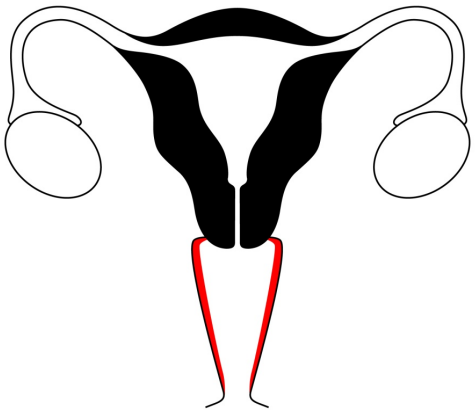
In the Western countries, the incidence of cervical cancer is decreasing, but is still one of the most common cancers affecting young and middle-aged women [1]. Advances in treatment of radiotherapy and additional chemotherapy have resulted in improvement in survival rates. Consequently, a rising number of cervical cancer survivors are at risk for chronic side effects. After treatment of cervical cancer, sexual dysfunction is a major symptom that affects women negatively. Still, vaginal pathophysiological changes and the effects of radiotherapy on the vaginal wall have received little attention.

The goal of cancer treatment is to cure with minimal morbidity. It is therefore of great importance not just to obtain tumor control, but also measuring morbidity after treatment to ensure the best possible quality of life for cancer survivors.

1.1 THE VAGINA

1.1.1 Anatomy

The vagina is a tubular organ extending from the uterus to the vestibulum. The vagina is developed by a prenatal fusion of the Müllerian ducts starting in gestational week 6. The fusion line can be seen as a longitudinal fold, columnae rugarum on the anterior and posterior wall. The average length is about 8-10 cm [2]; the front wall is slightly shorter than the posterior wall. Normally the vagina is collapsed with the front wall against the back wall. During sexual stimulation, the shape is changed and the back wall is extended. From the columnae rugarum originate horizontal folds, rugae vaginalis, which gives the vagina its great elasticity, a condition important at sexual intercourse and during childbirth. The vaginal wall has the following layers - the mucosa facing the lumen, the tunica muscularis comprising an inner circle of smooth muscle surrounded by a thicker outer longitudinally muscle layer and the exterior adventitia, a layer of connective tissue. The outer layer is rich in blood vessels, nerves and lymph vessels and shares part of the adventitia with the surrounding structures [3].



1.1.2 Blood supply

The vagina is richly vascularized and supplied by the vaginal arteries, branches of the uterine artery, middle rectal artery and the internal pudendal artery, all branches of the internal iliac arteries. A plexus of the vaginal veins is formed and drains into the internal iliac veins. The lubrication depends directly on the vessels, where an increased perfusion results in an increased passage of transudate through the vessel wall. The vessel function is controlled by the autonomic innervation [4].

1.1.3 Innervation

The innervation of the female genital tract is a delicate interplay between the autonomic nerves, both of sympathetic and parasympathetic origin, involving the pudendal nerve, the hypogastric plexus and the pelvic splanchnic nerve. The vaginal nerves are essentially derived from the uterovaginal plexus. The dorsal nerve of the clitoris is a branch from the pudendal nerve (S2-S4) and innervate the clitoris, labiae and the bottom third of the vaginal mucosa and the vulva. The hypogastric nerves innervate the upper 2/3 parts of the vaginal mucosa and control lubrication by regulating the blood flow. In men, who have undergone surgery for prostate and rectal cancer, the hypogastric nerve branches are uncovered at nerve-sparing surgery to maintain erectile function. Nerve-sparing surgery is not yet the standard in radical hysterectomy, but has started to be performed at some clinics [4]. Other effects of peripheral neuropathy may be changes in the pelvic floor muscles and neurogenic bladder disorder. Damage to the sacral nerves and their peripheral branches can also be associated with dyspareunia (pain during intercourse), or loss of sensation in the lower genital tract [5]. The sensory innervation of the upper part of the vagina is sparse compared to the lower part, which is abundantly supplied with free nerve endings [6].

Several studies have evaluated the innervation of the vaginal wall by analyzing the staining of protein gene product 9.5 (PGP 9.5), a neural marker for peripheral nerves and ganglia [7]. In women with pelvic organ prolapse, a decreased number of subepithelial nerve fibers have been seen in the anterior wall [8], but there are conflicting results in studies evaluating the innervation in the posterior wall. Increased nerve fibers were seen in women with pelvic organ prolapse by using PGP 9.5 antibodies [9], but fewer nerve bundles when using antibodies to protein S100 [10].

1.1.4 Histology

Histologically, the vaginal wall consists of four layers; the epithelium (against the lumen), the subepithelial layer or lamina propria (connective tissue), the smooth muscle layer and the adventitia [11]. The epithelium and the lamina propria are usually referred to as the mucosa (Figure 1).

The epithelium is lined with a superficial non-keratinized, stratified, squamous epithelium, consisting of basal, parabasal, intermediate and superficial squamous cells. As the stratum corneum is missing also the top cells retain their cell nuclei. The epithelial thickness varies with the menstrual phase and age. After the proliferation phase the top layer of cells are removed during menstruation. During high estrogen levels in the follicular phase the

epithelial cells form glycogen, which gives the cells a characteristic pale appearance with rounded shapes, smaller nuclei and perinuclear clearing [12].

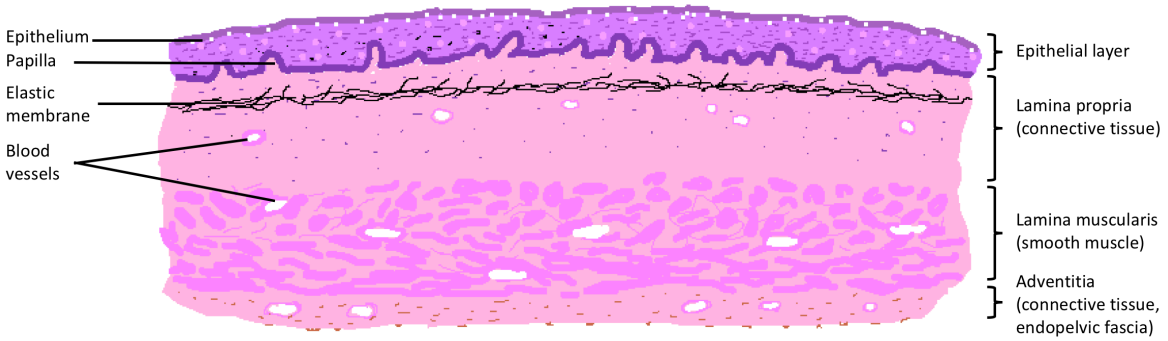


Figure 1. An illustration of the vaginal wall, with the mucosa (the epithelium and lamina propria), the smooth muscle layer and the adventitia. Illustration by Bo Blomgren.

Under the epithelium is the subepithelial layer, or the lamina propria, composed of a connective tissue layer with collagen and elastic fibers that are surrounded by an extracellular matrix (ECM). It is moderately cellular, with fibroblasts and smooth muscle cells and contains numerous capillaries and small lymph vessels. The basal membrane zone, between the epithelium and the underlying connective tissue, is formed with wrinkles. In healthy women before menopause numerous dermal papillae, consisting of connective tissue with capillaries, project into the epithelium supplying the epithelial cell layer. If the epithelium loses its dermal papillae, the epithelium will become thinner as necessary nutrients and oxygen supply no longer can reach the upper cell layers. The vaginal mucosa is one of few epithelia in the body, which are free of glands. The smooth muscle layer consists of an inner circular and an outer longitudinal layer. Finally, the adventitia surrounds the muscle layer, a loose connective tissue containing large venous plexus [11].

1.1.4.1 Biochemical aspects of the vaginal connective tissue

Elastin and collagen are the components that regulate the biomechanical properties of the vaginal wall. Elastin fibers provide elasticity and collagen fibers are very rigid. The ECM is constant remodeling, mediated by matrix metalloproteinases (MMPs), growth factors and signaling issued from the ECM itself [7]. Elastic fibers consists of an elastin core surrounded by proteins, such as fibulin-5, which promote elastogenesis and is crucial for the assembly of normal elastic fibers [13]. Fibulin-5 regulates the activity of the enzyme metalloproteinase 9 (MMP9) [14].

Collagen I, III and V are the main collagen subtypes in the vagina [15]. The collagen molecules, the tropocollagens, are made of three protein chains, coiled together to form a triple-helix [16]. Collagen I forms large and strong fibers, and collagen III and V form smaller fibers. The strength of the tissue depends on the proportion of each subtype and the cross-links that bind the tropocollagens together.

1.2 CERVICAL CANCER

1.2.1 Epidemiology

Cervical cancer is globally the fourth most common cancer in women, after breast cancer, colorectal cancer and lung cancer. In some developing countries in Eastern and Middle Africa it remains the most common cancer. The latest statistics from 2012 showed 528,000 new cases and 266,000 deaths from cervical cancer worldwide, accounting for 7.5 % of all female cancer deaths. Almost 9/10 (87 %) of cervical cancer deaths occur in less developed regions, where it accounts for around 12 % of all female cancers. High-risk areas include Eastern, Middle and South Africa and the Melanesia (Figure 2) [17].

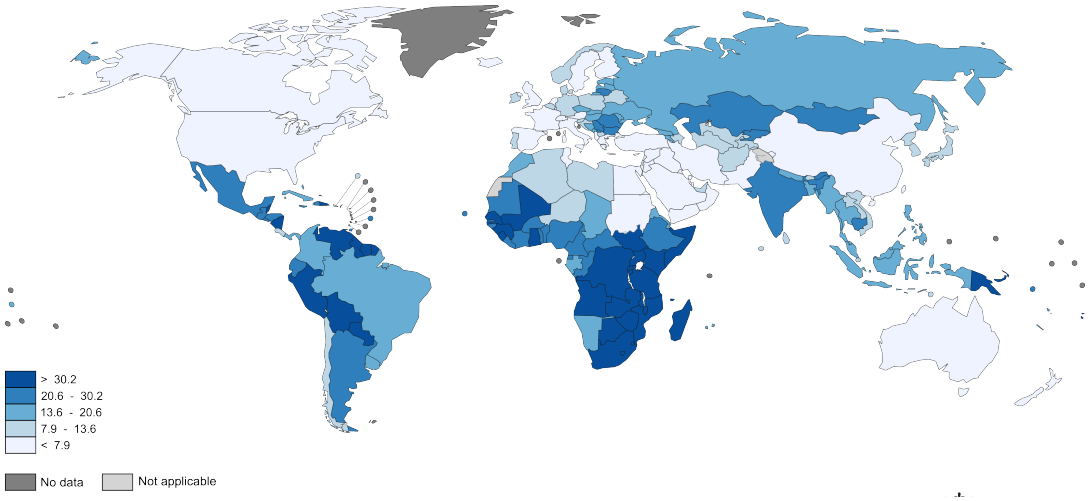


Figure 2. Estimated cervical cancer incidence worldwide 2012 (GLOBOCAN 2012, www.globocan.iarc.fr).

Gynecological cancer affects around 3,000 women per year in Sweden, of which cervical cancer represented 549 new cases in 2014 [18]. In Sweden the incidence of cervical cancer has decreased during the last 50 years, as a result of the organized screening program [19, 20]. However, during the last years the incidence has slightly increased (Figure 3).

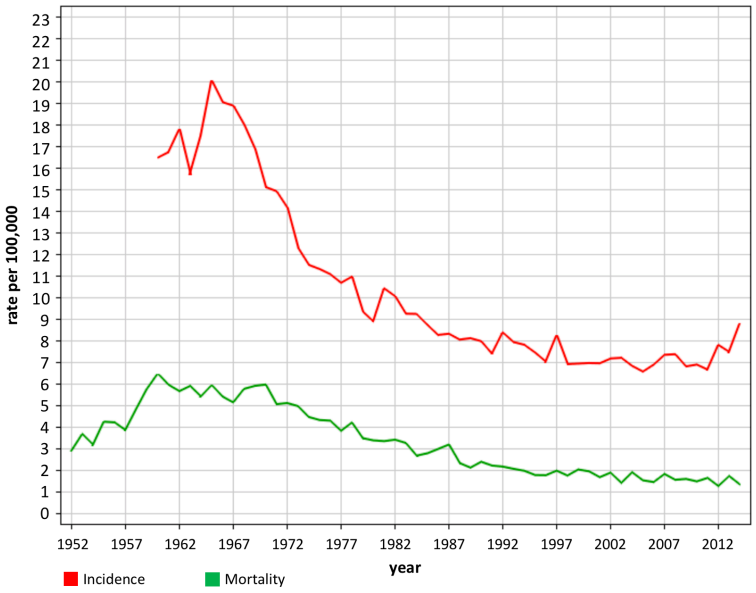


Figure 3. Cervical cancer incidence and mortality, Sweden 1952-2014 (NORDCAN database, www-dep.iarc.fr).

Cervical cancer primarily affects young and middle-aged women; the average age is around 50 years, about 30 % are under 40 years and 10 % are under 30 years. In Sweden, the mortality rate is low, especially in the younger women (Figure 4).

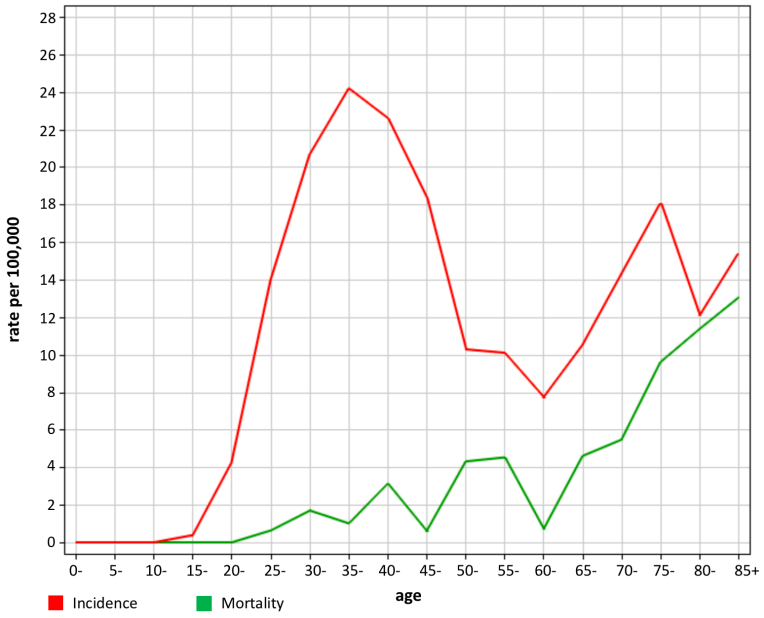


Figure 4. Cervical cancer age-specific incidence and mortality, Sweden 2014 (NORDCAN database, www-dep.iarc.fr).

1.2.2 Etiology and prevention

Cervical cancer is a sexually transmitted disease caused by a persistent infection with high-risk human papilloma virus (HPV) [21]. The oncogenic types 16 and 18 are the most common cancer-causing HPV types worldwide, found in about 70 % of all cases of invasive cervical cancer [22]. HPV infection is common in sexually active populations and

the majority of the HPV infections heal spontaneously within two years [23]. Only very few women develop a persistent HPV infection, implying the involvement of cofactors, which are still not fully known. Smoking is a known risk factor. Nicotine and other degradation products from smoking ends up in the mucus secreted by the glands of the cervix and accumulate on the epithelium [24]. Other known risk factors are multi-parity, concomitant genital infections, use of oral contraceptive and immunosuppression [25]. There is no proven hereditary form of cervical cancer, but as the immune system is involved genetic factors may play a role [26].

In Sweden, the screening program with Papanicolaou (Pap) smear started in 1966 and since 1977 there is a functioning program in all county councils [27]. The National Board of Health and Welfare (Socialstyrelsen) guidelines from 2017 form the basis for the screening program in terms of test method, age limits and intervals. The screening program, including HPV-testing, has shown a 60-70 % greater protection against invasive cervical cancer compared to cytology alone [28]. Women in Sweden are today offered liquid-based cytology between 23 and 64 years of age, and all women older than 30 years are tested for HPV. In 2015 the screening age was extended from 60 to 64 years of age. Testing is offered every third year for women between 23-49 years of age, and thereafter every seventh year [29]. For increasing participation in the cervical cancer screening, programs with a HPV self-test have been suggested for non-participants [30].

Organized Pap smear screening reduces the cervical cancer incidence [31] and HPV vaccination programs will hopefully further reduce the incidence in the future. The vaccines available today are all protecting for the two high-risk HPV types 16 and 18, showing protection against precancerous lesions [32]. A quadrivalent HPV vaccine also protects for HPV 6 and 11, which cause benign condyloma [33, 34]. A nine-valent vaccine adds protection for HPV 31, 33, 45, 52 and 58. The first vaccination is recommended before the sexual debut. In Sweden, the HPV vaccine has been subsidized for 10-12 years old girls in the National Vaccine program since 2012. The vaccines are prophylactic and the introduction has not changed the recommendation in the organized screening programs [35].

1.2.3 Clinical signs, diagnosis and staging

Women with early-stage cervical cancer often show no clinical signs. When clinical symptoms are present, the most common are postcoital bleeding, irregular bleeding and vaginal discharge. The diagnosis is based on histology of biopsies. Staging of cervical cancer according to definition from International Federation of Gynecology and Obstetrics (FIGO) is based on clinical examination performed during anesthesia [36] (Table 1, Figure 2). Lymph node status is not taken into account in this staging system. Since lymph node status is an important prognostic factor [37], it has recently been decided that the international recommendation will change to the use of the TNM (Tumour, Nodes, Metastasis) staging system in the future (Table 1).

According to the Swedish national guidelines for the radiological basis for staging of cervical cancer, examination with magnetic resonance imaging (MRI) of the pelvis and

computed tomography (CT) of the thorax and abdomen are recommended, except for cervical cancer stage IA1. Additional positron emission tomography (PET) scan is recommended in women planned for primary definitive radiochemotherapy and in all cases of neuroendocrine histology [38].

1.2.4 Histology

The squamous cell carcinoma is the most frequent histological type and accounts for 80-90 % of the cases of cervical cancer, followed by adenocarcinoma (10-20 %) and adenosquamous carcinoma (3-5 %) [39]. In Western countries, the relative proportion of cervical adenocarcinoma has increased over the last 50 years [40, 41]. One possible explanation is that the screening methods are more effective detecting squamous cell cancer compared with adenocarcinoma [42]. Glassy cell carcinoma and neuroendocrine small cell carcinoma are more unusual subtypes, with poorer prognosis [43, 44].

Table 1. Stages of cervical cancer according to FIGO nomenclature [36] and the TNM staging system [45].

TNM	FIGO	
Primary tumor (T)		
TX		Primary tumor cannot be assessed
T0		No evidence of primary tumor
Tis		Carcinoma in situ
T1	I	Cervical carcinoma confined to the uterus (extension to the corpus should be disregarded)
T1a	IA	Preclinical invasive carcinoma, diagnosed by microscopy only
T1a1	IA1	Minimal microscopic stromal invasion (≤ 3 mm stromal invasion (in depth), ≤ 7 mm in horizontal spread)
T1a2	IA2	Tumor with an invasive component more than 3 mm and not more than 5 mm and 7 mm or less in horizontal spread
T1b	IB	Clinical lesions confined to the cervix or preclinical lesions greater than stage IA
	IB1	Clinical lesions no greater than 4 cm
	IB2	Clinical lesions greater than 4 cm
T2	II	Cervical carcinoma invades beyond the uterus but not to the pelvic wall or to the lower third of the vagina
T2a	IIA	Tumor without parametrial invasion
	IIA1	Tumor less than 4 cm with involvement of less than the upper two-thirds of the vagina
	IIA2	Tumor greater than 4 cm with involvement of less than the upper two-thirds of the vagina
T2b	IIB	Tumor with parametrial invasion
T3	III	Cervical carcinoma extends to the pelvic wall and/or involves the lower third of the vagina and/or causes hydronephrosis or nonfunctioning of the kidneys
T3a	IIIA	Tumor involves the lower third of the vagina with no extension to the pelvic wall
T3b	IIIB	Tumor extends to the pelvic wall and/or causes hydronephrosis or nonfunctioning of the kidneys
T4a	IVA	Tumor invades the mucosa of the bladder or rectum and/or extends beyond the true pelvis
Regional lymph nodes* (N)		
NX		Regional lymph nodes cannot be assessed
N0		No regional lymph node metastasis
N1		Regional lymph node metastasis
Distant metastasis (M)		
MX		Presence of distant metastasis cannot be assessed
M0		No distant metastasis
M1	IVB	Distant metastasis

*Regional lymph nodes include paracervical, parametrial, hypogastric (obturator), common, internal and external iliac, presacral and sacral.

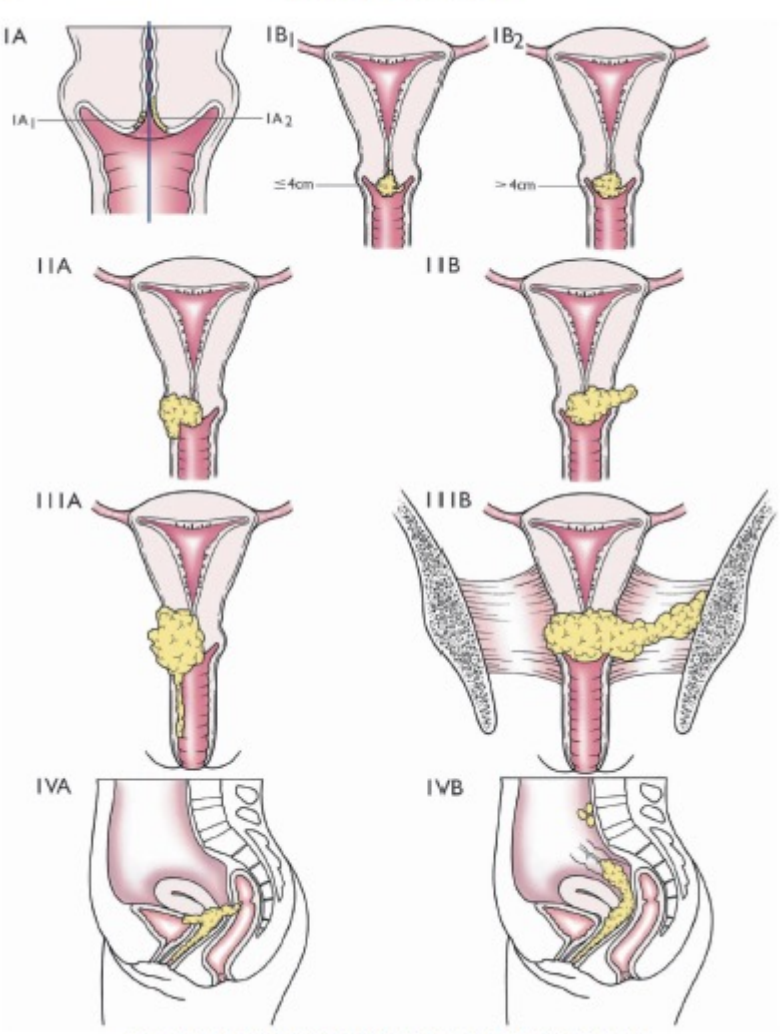


Figure 5. Staging of cervical cancer according to FIGO, stage IA1-IVB (from Quinn el al. [46]).

1.2.5 Treatment

The treatment of cervical cancer can include surgery, radiation and chemotherapy depending on the stage at diagnose. Microinvasive cervical cancer (FIGO stage IA1) is usually cured by simple hysterectomy or cone biopsy. In stage IA2 or IA1 with lymphovascular space involvement (LVSI) radical hysterectomy or trachelectomy, with additional pelvic lymph node dissection is recommended [38, 47, 48]. Surgery and radiotherapy are reported to be equally effective in cervical cancer stage IB1 and IIA1 [49]. Primary radical surgery with radical hysterectomy and pelvic lymph node dissection is usually the choice of treatment due to the risk of side effects by radiotherapy. Primary surgery is not recommended in patients with preoperatively diagnosed large tumors (more than 4 cm) or lymph node metastasis, since treatment including radical hysterectomy and radiotherapy have shown to give more side effects, compared to treatment with surgery or radiotherapy alone [50, 51]. In patients with unfavorable prognostic factors after surgery, such as lymph node metastasis and positive resection margins, adjuvant chemoradiation has shown to increase the progression-free survival at 4 years from 63 to 80 % and overall survival from 71 to 81 % compared with radiotherapy alone [52].

Previously, preoperative intracavitary brachytherapy (BT) was often used at Karolinska University Hospital for early stage of cervical cancer (at that time stage IB-IIA). The treatment included two intracavitary radiotherapy applications with a three-week interval, followed by surgery [53]. Currently, standard treatment for locally advanced cervical cancer (FIGO stage IB2, IIA2-IVA) includes external beam radiotherapy and intracavitary radiotherapy combined with weekly intravenous cisplatinum [54]. In stage IB2, IIA2 and IIB the risk for lymph node metastasis are reported to be between 19 to 44 % [55, 56], resulting in the recommendation of primary definitive radiochemotherapy.

1.2.5.1 Surgery

Standard radical surgery for cervical cancer stage IA2, IB1 and IIA1 includes radical hysterectomy with complete resection of the parametria and the paracervical tissue, pelvic lymphadenectomy and resection of the proximal portion of the vagina [57]. For early stages (IA2, IB1) radical trachelectomy and pelvic lymphadenectomy can be an alternative treatment for preserving fertility. For safety this option requires tumor size less than 2 cm and no high-risk tumor histology [58].

Primary radical surgery can be performed by laparotomy or robot-assisted laparoscopic surgery. Studies on robot-assisted laparoscopic surgery indicate less per-operative bleeding and shorter hospital stay with fewer complications compared to open surgery [59-62]. Still, today laparotomy is considered the gold standard surgical management, due to the lack of randomized clinical trials [63, 64]. Ovarian preservation is recommended in premenopausal women, except in cases of larger adenocarcinoma (more than 2 cm) [65].

Nerve-sparing surgical techniques have shown to improve postoperative quality of life, with less impaired bladder function [66] and less impaired sexual function [67].

1.2.5.2 Radiotherapy

Definitive radiochemotherapy for cervical cancer including external beam radiotherapy (EBRT), intracavitary radiotherapy (brachytherapy) and concomitant weekly cisplatin is standard treatment for cervical cancer stage IB2, IIA2-IVA [54]. EBRT over a pelvic field includes the internal and external iliac nodes, the lower common iliac nodes up to the level of the space between the lumbar vertebrae L4 and L5. The treatment is usually delivered with a daily fraction of 1.8-2.0 Gy to a total dose of 45-50 Gy.

Brachytherapy is of great importance for the success of the cervical cancer treatment [68, 69]. Until the last decade, the intracavitary BT dose was prescribed to the “Point A”, located 2 cm proximal from the mucous membrane of the lateral fornix and 2 cm lateral of the central axis of the uterus, introduced by the Manchester system [70]. Today, MRI-based 3D treatment planning of the BT is recommended, according to European recommendations [71-74] and American guidelines [75-78]. In MRI-based image guided adaptive brachytherapy (IGABT), with repeated volumetric imaging, the treatment planning adapts to the reduced tumor volume over time. Studies have shown both reduced normal tissue

toxicity and better local tumor control [79, 80]. Treatment combining the intracavitary BT with interstitial BT, in which needles are inserted to the tumor has shown both superior target coverage and organ sparing, compared to intracavitary BT alone [81]. BT can be delivered by different dose rates. High dose rate (HDR) treatment is the most frequently used worldwide. In Sweden, today, HDR or pulsed dose rate (PDR) are the used techniques. PDR delivers the HDR in a number of fractions during a longer period of time, which radiobiologically resembles LDR (low dose rate).

A total radiation dose to the high-risk clinical target volume (HR-CTV) of $D_{90\%} > 85$ Gy, converted to biologically equivalent dose in 2 Gy fractions (EQD_2), is considered necessary for local tumor control [82, 83]. A more recent retrospective study has shown that $D_{90\%} \geq$ 87 Gy to the HR-CTV reduced the risk for local recurrence from 20 % to 4 % [84]. Modern radiotherapy modality like intensity modulated radiation therapy (IMRT), gives a more individually optimized treatment [85].

Concomitant chemotherapy during the radiotherapy, with weekly cisplatin, is given to enhance the radiosensitivity in the tumor cells [86]. It is today a standard treatment based on meta-analysis showing a 6 % improvement in the 5-year survival rate, compared to radiotherapy alone [87].

Adjuvant radiochemotherapy after radical surgery is indicated in cases of unfavorable prognostic factors, such as lymph node metastasis, positive surgical margins, tumor growth in parametria, tumor size more than 4 cm, LVSI, growth in the outer 1/3 of the stroma or small cell neuroendocrine histology [38, 88, 89]. Standard treatment is EBRT to a pelvic field with a minimum dosage of 45 Gy, and five courses of concomitant weekly cisplatin. In cases of large tumor size and/or close surgical margins, vaginal boost given to the upper 1/3 part of the vagina, should be considered [38].

1.2.6 Survival and prognostic factors

The prognosis of patients with cervical cancer is related to tumor stage. In the FIGO 26th annual report including data from 37 countries, with 11,775 women treated 1999-2000, the stage was the most important prognostic factor [46]. Women diagnosed in stage IA1 had a 5-year survival of 98 % compared to 9 % for women in stage IVB. Other important prognostic factors are lymph node status and tumor size [46].

In Sweden the overall 5-year survival rate for cervical cancer is 73 % reported in 2014 [18]. A majority of patients diagnosed with cervical cancer are young or middle-aged women with a long life expectancy and will live many years with the chronic side effects of the treatment.

1.3 SEX STEROID HORMONES IN WOMEN

Estrogen, progesterone and androgen, the sex steroid hormones, are mainly synthesized in the gonads and the adrenal cortex. The system of female hormone production consists of different levels, the hypothalamus, the central regulator, through the hypothalamic-pituitary-ovarian and the hypothalamic-pituitary-adrenal axes. From the anterior pituitary gland follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are released after stimulation by gonadotropin releasing hormone (GnRH) from the hypothalamus. The ovarian hormones estrogen and progesterone are secreted in response to FSH and LH [90].

The main site of estrogen synthesis differs between pre- and postmenopausal women. In premenopausal women, estrogens are mainly produced in the granulosa cells of the ovary during the menstrual cycle, while lower levels of estrogen are also produced in the bone, muscle, fat and vascular tissue and the brain [91]. In the follicular phase the secretion of FSH rises whereas LH remains low. FSH stimulates the growth of follicles in the ovaries leading to increased estradiol production. Via a feedback loop a high level of LH is established, which induces ovulation. After ovulation estradiol levels fall and the corpus luteum is formed. In the luteal phase, the corpus luteum produces estradiol and progesterone, which have an inhibitory effect on the secretion of FSH and LH. If fertilization does not occur, the corpus luteum degenerate and the secretion of sex steroid hormones drop, which initiates the menstrual bleeding.

Menopause is defined as the time when there has been no menstrual period for one year, with no other identified biological or psychological cause. Natural menopause is the physiological transition when the ovulations stop, the cyclic ovarian secretions of female sex steroid hormones disappear and the menstrual periods finally end. Consequently, the level of FSH will increase. The mean age of menopause in Sweden is 51 years [92]. Iatrogenic early menopause can be caused by cancer treatment, with radiotherapy, chemotherapy or by surgery [93]. In postmenopausal women the main sites of estrogen synthesis are the adipose tissue, bone, liver and brain [94]. Estrogen synthesized in the extra gonadal sites acts mostly locally, in contrast to ovarian-synthesized estrogen produced in premenopausal women, which is mainly released into the circulation [95].

1.3.1 Estrogens

Estrogens are C18 steroids derived from cholesterol. The primary source of estradiol in woman is the granulosa cells of the ovaries. Estrogens are synthesized from androgens by the enzyme aromatase. The estrogen with most estrogenic activity is estradiol (E2, or 17β-estradiol); whereas estrone (E1) and estriol (E3) have much weaker activity. E2 is the most potent estrogen in premenopausal women, while E1 is more important in postmenopausal period. E1 is then synthesized from adrenal androstenedione in fat tissue. E3, the least potent estrogen, is formed from E1 in large amount during pregnancy by the placenta. For transportation in the blood, 69 % E2 is reversibly bound to sex-hormone-binding globulin (SHBG), 30 % bound to albumin and only 1 % is freely circulating [90]. SHBG binds with lesser affinity to estradiol, compared to its high affinity binding to testosterone [96]. SHBG is synthesized in the liver cells and both the natural estradiol and the synthetic ethinyl

estradiol increase the production [97]. In use of oral contraceptives with ethinyl estradiol, the plasma levels of SHBG rise by at least 5-fold [98], further decreasing the levels of the free testosterone [99]. In postmenopausal women using hormone replacement therapy (HRT), plasma levels of SHBG has been reported to increase following oral HRT, but were not affected by transdermal HRT [100]. If this finding has any clinical implication in HRT users is not known.

1.3.2 Steroid hormone receptors

The steroid hormone receptors are a subset of proteins within the nuclear receptor family that initiate signal transduction for steroid hormones, which lead to changes in gene expression in the target tissue [90]. Nuclear receptors are ligand-activated transcription factors that control the rate of transcription of genetic information from DNA to messenger ribonucleic acid (mRNA). These receptor protein chains are folded into domains with specific functions [101].

The genomic pathway mediates the transcription of gene segments to mRNA in the nucleus and the subsequent translation to specific proteins in the target tissues, within hours. The non-genomic pathway, with rapid effects (within seconds) involves the interaction with membrane recognition site of the steroid hormone [102].

The estrogen receptor (ER) has two subtypes, ERα and ERβ. The presence of ERα was discovered in 1958 [103] and in the 1990s the ERβ was identified [104]. The two isoforms may explain some of the different estrogen responses in different tissues. They have similarity in ligand binding affinity, but appear to activate transcription differently [105]. The ERs have the highest affinity to estradiol, less to estrone and ever weaker to estriol. ERα and ERβ share many functional characteristics, but the regulation of transcription activity and the tissue locations are different. The roles of these receptors have been clarified in female reproduction by studying knockout mice [106]. ERα is suggested to have proliferative effects in most tissues, whereas ERβ has an anti-proliferative and pro-differentiative effect [107]. The tissue distribution of the ERs is overlapping. ERα is present in the urogenital tract, breast, brain, cardiovascular system, liver and in bone. The ERβ subtype has been identified in the urogenital tract, kidney, brain, bone, heart, lungs, gastrointestinal tract and endothelial cells [107].

The progesterone receptor (PR) exists in two isoforms, A and B that originate from the same gene. PRA and PRB are almost identical, but PRA is shorter, lacking 164 amino acids, compared to PRB. The PR and ER expression in the human endometrium increases during the follicular phase and decreases during the luteal phase of the menstrual cycle [108, 109], but in the outer portion of the uterine wall a cyclic pattern of ER and PR expression is not found [110]. In postmenopausal women without HRT, high stabile expression of both ER and PR were found in all the uterine layers, suggesting that the expression of ER and PR is essential for the uterine tissue [110]. In the luteal phase, when the progesterone level is high, ER expression in the human breast tissue is down regulated, while PR expression in the breast remain high throughout the normal menstrual cycle. However, under influence of exogenous gestagens both ERs and PRs

are down regulated [111], indicating the complexity of the steroid receptor expression being affected of both the hormonal status and the target tissue.

The androgen receptor (AR) exists in a long A-form and a shorter B-form. The AR expression has been documented in numerous tissues, including the central nervous system, urogenital tract, breast, bone, muscle and cardiovascular system. The AR is most closely related to the PR and progestin (synthetic progesterone) in high dosage can block the AR [112]. Consequently, progestin may act as an antiandrogen as well as an antiestrogen.

In female genitals the expression of ERα and PR gradually decrease from the vagina to the vulva, whereas the highest concentration of AR is found in the vulva [113]. ERα and ERβ mRNAs have been demonstrated in extracts from vaginal tissue, with the highest level of expression in premenopausal women. Postmenopausal women receiving HRT showed the lowest mRNA ERα expression. No differences were seen in ERβ expression between postmenopausal women with or without HRT [114].

In pre- and postmenopausal women with prolapse, IHC showed higher expression of ERα in the premenopausal group both in the anterior and the posterior walls, with a positive correlation to the concentration of blood vessels [115]. In one study, treatment with local estrogen therapy was leading to increased ERα and PR expression in the posterior wall in postmenopausal women with prolapse, while ERβ expression was almost unchanged [116]. Table 2 presents previous studies on sex steroid hormone receptor expression in the vaginal wall. There are no studies on the steroid receptor expression in the vaginal wall after treatment of cervical cancer with radiotherapy.

Table 2. Studies on sex steroid hormone receptor expression in the vaginal wall.

	Study objects	Study design	Results
Van Haaften, 1997 [117]	Women (n=29)	ERc, PRc and ERn in the endometrium, myometrium and vagina in postmenopausal women treated with vaginal estriol (0.5 mg daily) compared to 17β-estradiol (0.05 mg daily) therapy.	No differences between vaginal estradiol and estriol medication with regard to the effects on receptor levels in vaginal and uterine tissues.
Chen, 1999 [118]	Women planned for hysterectomy	ERα, ERβ mRNA expression in premenopausal (n=12) and postmenopausal women (n=4)	ERβ was absent in the vaginal walls of postmenopausal women. ERα were found in both groups.
Gebhart, 2001 [114]	Women (n=75)	ERα, ERβ mRNA expression in premenopausal (n=25), postmenopausal with estrogen replacement therapy (ERT) (n=25) and postmenopausal without ERT.	Postmenopausal lower ERα expression compared with premenopausal, but no difference between postmenopausal with ERT compared to premenopausal. Postmenopausal lower ERβ expression than premenopausal regardless of ERT.
Pessina, 2006 [119]	Rats	IHC ERα, PR, AR in the vaginal wall was analyzed after oophorectomy, and treatment with estradiol, testosterone and progesterone.	Oophorectomy resulted in an increase in ERα and a decrease in PR. Estradiol down-regulated ERα and up-regulated PR expression. AR unaffected.
Söderberg, 2007 [120]	Women with stress urinary incontinence (SUI)/controls	ERα, ERβ, PR, AR mRNA expression in women with SUI, premenopausal (n=4), postmenopausal (n=8). Controls premenopausal (n=6), postmenopausal (n=5)	Higher ERβ in the premenopausal women with SUI, compared to controls. Lower PR scores in the postmenopausal groups.
Baldassarre, 2013 [121]	Women with prolapse and women with testosterone treatment	AR mRNA expression and IHC AR in mucosa and stroma in premenopausal (n=16), postmenopausal (n=16) and T-treated women (n=16).	Higher AR protein and mRNA expression in premenopausal compared to postmenopausal. Higher AR protein and mRNA expression after testosterone (T) administration.
Lara, 2014 [115]	Women with prolapse	IHC ERα and endothelial cell marker CD3 to label vessels in premenopausal (n=6) and postmenopausal women (n=6).	Postmenopausal lower ERα expression, compared with premenopausal. A positive correlation between ERα and the vessel concentration.
Fuermetz, 2015 [116]	Women corpses and biopsies	IHC ERα, ERβ, PR in premenopausal corpses (n=60), postmenopausal corpses (n=43) and postmenopausal women treated with local estrogen therapy (n=80)	ERα expression was higher in the epithelium and stroma in the postmenopausal women with local estrogen therapy. PR expression was increased in the group with local estrogen therapy. ERβ expression was unchanged.
Sawczuk, 2017 [122]	Women (n=60), mean age in the three groups 53-60 years	IHC ERα, ERβ in smears of vaginal mucosa after using po estradiol (1 mg) therapy (n=20), transdermal estradiol (0.5mg +NETA) therapy (n=20) and vaginal estradiol 0.5 mg (n=20) in 3 months.	Highest increase in intensity of ERα after topical therapy, lowest after oral therapy.

1.4 LATE SIDE EFFECTS IN CERVICAL CANCER TREATMENT

Chronic side effects can either develop directly after acute toxicity or years after completed cancer treatment. Radiotherapy-induced acute radiation damage starts after exposure and symptoms develops gradually during the treatment course. Reactions with symptoms presented more than three months after completed radiotherapy are considered to be late side effects [123, 124]. The degree of the radiation related toxicity depends on treatment factors such as total dose, dose per fraction, total treatment time, irradiated volume and type of radiation. Patient-related factors as comorbidity, smoking, age and additional treatments as surgery and chemotherapy may worsen the late side effects [125-127]. Vascular damage, fibrosis and neural damage are tissue response in the complex radiation injury [128, 129]. Acute vascular changes result in cellular damages, increased permeability and inflammation, which leads to vascular injury and hypoxia, causing chronic tissue damage [130]. This cascade of cytokine activation and further oxidative stress creates a dynamic process promoting collagen formation [131]. Better knowledge in these mechanisms hopefully could lead to new approaches towards prevention of side effects [132].

Radical hysterectomy with pelvic lymph node dissection for early cervical cancer can be associated with long-term morbidity with sexual, bladder and bowel dysfunction [133]. Hopefully, new surgical nerve-sparing techniques will reduce the postoperative side effects, but is not yet the standard in all centers [4].

The late side effects of radiotherapy on the surrounding pelvic structure, with morphological changes and symptoms on bladder and intestine, are well documented [134, 135]. The cancer survivors report gastrointestinal symptoms of diarrhea, defecation urgency, fecal incontinence and abdominal pain after pelvic radiation [136-138], symptoms associated with distress [139]. Bleeding from the bowel, fistulas between vagina and bladder or rectum are examples of severe late side effect after radiotherapy for cervical cancer [140]. Urinary symptoms with voiding difficulties and urinary incontinence can occur postoperatively after radical hysterectomy or after combination treatment with surgery and adjuvant radiotherapy [141]. Urological late side effects after radiotherapy are hematuria, dysuria, shrunken fibrotic bladder, ureteral stenosis and fistulae [142].

1.4.1 Sexual dysfunction after cervical cancer treatment

Women who have been treated for cervical cancer often report persistent changes in their sexual function, which results in considerable distress and decreased quality of life [143]. The World Health Organization define sexual health as a state of physical, emotional, mental and social well-being in relation to sexuality [144]. Female sexual dysfunction refers to persistent problems with the sexual response cycle; desire, arousal and orgasmic phase or sexual pain, that results in personal distress [145]. Epidemiological studies of the general population in the United States have shown prevalence of sexual dysfunction in women to approximately 40 % [146, 147]. The prevalence among gynecological cancer survivors differs in previous reports from 48 % up to 90 % [148-150]. The difference can be due to methodological variations, heterogeneity regarding patients' groups and/or the use of various questionnaires.

Cervical cancer is often diagnosed in young or middle-aged women, who are usually sexually active [151]. The gynecological tumor and the treatment with surgery, radiation and/or chemotherapy directly affect the reproductive and sexual organs. The sexual dysfunction in cervical cancer survivorship could start directly after treatment [152] and persist in long-term survivors [153, 154]. Women treated with radiotherapy have shown worse sexual functioning compared to survivors treated with surgery alone [155, 156]. There are reports on sexual function after radical hysterectomy alone showing no adverse impact [155]. However, other studies have shown negative effects, such as insufficient lubrication, reduction in vaginal length and elasticity [143]. In a large longitudinal study, persistent negative impact on sexual interest and vaginal lubrication was found, but the other sexual problems, such as orgasmic difficulties, reduced vaginal size and dyspareunia ended over time [157].

Cervical cancer and its treatment can affect the different stages in the sexual response cycle [158]. Desire has been reported to be decreased after different treatment modalities, such as radical hysterectomy [157] and treatments including radiotherapy [159, 160]. The prevalence of decreased lubrication when sexually aroused have been reported after surgery alone [161], after combined treatment with surgery and radiotherapy and after primary radiotherapy [143, 162, 163]. Vaginal dryness of various degrees has been reported after all treatment modalities [161, 164-166]. Reduced frequency of orgasm is reported after treatments with surgery and radiotherapy [155, 157] and after primary radiotherapy [162], but there are also reports where no differences were found between cancer survivors and controls in this aspect [143, 165, 167]. A higher prevalence of dyspareunia has been reported in several studies comparing cervical cancer survivors versus healthy control women [143, 157, 162, 165, 167]. Significantly more dyspareunia in cervical cancer survivors treated with surgery and radiotherapy, compared to survivors treated with surgery or radiotherapy alone, where found by Park *et al*. [167]. In contrast, Pieterse *et al.*, found no increased risk of dyspareunia of adjuvant radiotherapy following surgery [165].

The pathophysiology of the genital symptoms and sexual dysfunction in cervical cancer survivors can be explained by multiple factors. Radical hysterectomy can cause pelvic autonomic nerve damage [168, 169], which may affect the neural vascular control with genital swelling and lubrication response during sexual arousal [170]. Surgery also causes a direct shortening of the vagina and risk of postoperative fibrosis in the proximal part. Fibrosis and vascular changes can also be caused by radiotherapy [171]. Collagen deposition is described as a part in the defect tissue healing in other irradiated tissues [128, 172]. The hormonal changes with reduction in estrogen as a consequence to oophorectomy or radiotherapy can lead to thinning of the vaginal wall and attenuated genital blood flow [173]. Psychological factors can affect sexuality in cancer survivors by stress, anxiety, depression and negative sexual body image [174, 175].

1.4.2 Vaginal changes after radiotherapy in cervical cancer survivors

Vaginal stenosis after radiotherapy, defined as an abnormal vaginal tightening and shortening due to the formation of fibrosis, is a common late side effect in cervical cancer

survivors [176]. In the literature, there is a variation in prevalence from 38 % to 88 % [177-180]. The variation can be due to tumor-, treatment- and patient-related factors.
The interpretation of previous studies is further complicated by the use of different grading systems. In the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) v.3.0, vaginal stenosis was defined as vaginal narrowing and/or shortening; not interfering with function (grade 1), interfering with function (grade 2) and as complete obliterated (grade 3). In the new version CTCAE v4.0, vaginal stenosis is replaced with vaginal stricture (characterized by narrowing of the vaginal canal); asymptomatic, mild (grade 1), narrowing and/or shortening not interfering with examination (grade 2), narrowing and/or shortening interfering with sexual activity or examination (grade 3). Other grading systems for morbidity after radiotherapy are the Franco-Italian Glossary [181] and the Late Effects in Normal Tissues-Subjective, Objective, Management and Analytic Score (LENT-SOMA) [182]. Kirchheiner *et al*. have described different degrees of vaginal changes based on vaginoscopy images [183].

Radiation induced injuries in capillaries with dilatation can manifest as telangiectasia, abnormal superficial vessels in the mucosa, which can bleed during sexual intercourse and at gynecological examination. Telangiectasias, as well as the signs of atrophy, are typical findings at gynecological follow-ups [183]. However, these findings are rarely graded in the clinical situation. Heavy smoking (>10 cigarettes per day) during radiochemotherapy is associated with severe late side effects, with a 3 % prevalence of total vaginal necrosis [184].

There are only a few previous studies analyzing vaginal biopsies after radiotherapy in cervical cancer survivors. Pitkin and Bradbury analyzed in 1965 punch biopsies from the vaginal fornix in women after radiotherapy, finding that thickness and maturation of the epithelium increased over time, reaching a maximum after two years. They also reported signs of epithelial regeneration in women treated with estrogen cream [185]. In contrast, Abitbol el al found no changes in vaginal cytology after women had been treated with systemic estrogen therapy [186]. In vaginal biopsies after radiotherapy, atrophic mucosa as well as hyalinization and collagenization of the connective tissue, was shown [186]. In 1995 Shield showed similar findings in vaginal biopsies after radiotherapy with atrophy and stromal changes with fibrosis, vessel changes and atypical fibroblasts [187].

In a placebo-controlled trail by Pitkin *et al*., women reported less vaginal bleeding and dyspareunia when treated with estrogen cream (0.01 % dienestrol) for 3 months, compared to women with placebo cream [188]. Table 3 presents previous studies on changes in the vaginal wall after radiotherapy in cervical cancer survivors, including studies on intervention with estrogen therapy and vaginal dilation.

There is limited data on the irradiation tolerance dose of the vagina [189, 190]. A proposal to report dose-effect relationship between the radiation dose to the recto-vaginal reference point and the vaginal morbidity has been made in the EMBRACE study [191]. A dose ≤ 65 Gy EQD2 to the recto-vaginal reference point was proposed for reducing the risk of vaginal stenosis ≥ grade 2 [191]. Further studies by Westerveld *et al*. have presented a method for reporting vaginal dose contributed of EBRT and BT along the vaginal axis [192]. They

further concluded that the dose at the mid vagina was reflecting the dose from BT, while the dose contributed to lower vaginal point was dependent of the EBRT field border location [193].

Table 3. Studies on changes in the vaginal wall after radiotherapy for cervical cancer; intervention with estrogen treatment and vaginal dilation.

	Study objects	**Study design**	**Results**
Vasicka, 1958 [180]	Women treated with RT (n=16), stage I-II, age <50 yrs	Pelvic examination	Vaginal stenosis 81 %
Pitkin, 1965 [185]	Women treated with RT (n=49), age <50 yrs, stage not stated, sexual intercourse > 1/month, 1d-3yrs after RT.	Cytological (vaginal smear) and histologic study (punch biopsies from the fornix). Treatment with estrogen cream (0.01 % dienestrol) for 3 months (n=31) vs. treatment with cream without estrogen (n=7).	The thickness and maturation of the epithelium increased over time after end of RT, with a maximum after 2 yrs. Treatment of estrogen cream had a significant effect on epithelial regeneration, especially in patients post-RT >3 months.
Griffin, 1968 [194]	Women treated with RT (n not stated)	Clinical examination	Fibrous connective tissue, telangiectasia, vaginal shortening, loss of functional vagina
Pitkin, 1971 [188]	Women treated with RT (n=93), stage I-IV	Double-blind, placebo-controlled, topical estrogen therapy (0.01 % dienestrol cream, 3 times/w, 5-8 months) (n=44) vs placebo (n=49), clinical examination	The estrogen-treated group had less vaginal bleeding and the vaginal epithelium was considered normal in appearance in 43 % vs 10 % in controls. Frequency of intercourse was equal, but the estrogen-treated group reported less dyspareunia.
Hartman, 1972 [179]	Women treated for cervical cancer (n=221), stage I-IV	Retrospective study, medical records	Vaginal stenosis 88 %
Abitbol, 1974 [186]	Women, at least 1 year after end of treatment for cervical cancer st I-II (n=97), 23-68 yrs Surgery alone (n=41) Primary RT (n=37) Comb (n=19)	Pelvic examination Gross appearance and vaginal cytology after RT (n=17) and after surgery alone (n=13). Vaginal biopsies after surgery (n=7). Vaginal cytology before and after systemic estrogen therapy (2.5 mg conjugated estrogen, Premarina®, daily for 1 week) in the group that had received RT (n=17).	After radiation narrowing or vaginal obliteration (81 %), pelvic fibrosis, telangiectasia. Atrophic mucosa, hyalinization and collagenization of the connective tissue, the muscular coat of vessels was thickened with fibrotic tissue, which replaced the muscular fibers. No change in vaginal cytology after estrogen therapy.
Pitkin, 1975 [195]		Letter to the Editor, discussing the study above by Abitbol.	

Poma, 1980 [196]	Women treated with RT with complete vaginal occlusion (n=5), stage II	Case report. Measuring the vagina, after digital pressure and topical estrogen therapy twice a day for 6-8 weeks.	The five women received a functional vagina, length 10 cm, at end of treatment.
Bruner, 1993 [178]	Women treated with RT for cervical cancer (n=42), and endometrial cancer (n=48), age 30-83 yrs	Vaginal length was measured, before treatment, 6-12 month, 1-2 years and >2 years after treatment	Vaginal stenosis 72 %. Vaginal length decreased over time, greater in patients treated for cervical cancer, and greater in patients with more advanced stage.
Shield, 1995 [187]	Women treated with RT (n=24) (9 were also treated with surgery). Age not reported.	Cervicovaginal smear Vaginal biopsies (n=11), collected 6 weeks to 4.5 years after RT.	Fibrosis, hyalinization, bizarre fibroblast in the stroma. Atrophy in 76 % of the biopsies. Smears showed atrophic pattern in 88 %, and large amount of "atypic cells".
Grigsby, 1995 [171]	Review	Review of late effects of cancer treatment of the female reproductive tract.	Grading system, tolerance doses, management strategies.
Decruze, 1999 [197]	Women treated with RT for cervical cancer (n=29), and endometrial cancer (n=41), age 35-87 yrs	Retrospective study evaluating use of vaginal dilator (n=35), with a historical control group not using dilator (n=35). Vaginal exam, 3-12 months after end of treatment.	Vaginal stenosis in 11 % of women in the group using a dilator, compared to 54 % in the controls.
Brand, 2006 [177]	Women treated with RT (n=188), stage I-IV, age 26-85 yrs	Retrospective study, medical records, follow-up 2.4-129 months.	Vaginal stenosis grade 1-2 in 38 % of the patients. Risk factor, age over 50 yrs.
Guth, 2010 [184]	Women treated with RCT (n=98), stage I-IV, age 26-84 yrs	Retrospective study, medical records, focus on severe late toxic reactions, total vaginal necrosis.	8.2 % severe late toxic reaction 3.1 % total vaginal necrosis, risk factor smoking (>10/d).
Kirchheiner, 2012 [183]	Women treated with primary RT +/- chemo (n=22), stage I-IV, age 35-76 yrs	Vaginoscopy with photo documentation 3-24 months after end of treatment.	Mucosal pallor, telangiectasia, fragility of the vaginal wall, ulceration, and adhesions/occlusion.

2 AIMS OF THE THESIS

The overall aim was to improve our knowledge of the chronic side effects in cervical cancer survivors treated with radiotherapy, with particular focus on vaginal morphological changes and sexual dysfunction.

The specific aims of the thesis:

- To investigate the morphology of the connective tissue of the vaginal wall in cervical cancer survivors treated with radiotherapy, with focus on the amount and structure of elastin and collagen fibers.

- To analyze the morphological changes in the vaginal epithelium in cervical cancer survivors treated with radiotherapy and its correlation to serum levels of sex steroid hormones and sexual function.

- To study the amount and distribution of sex steroid hormone receptor expression (both on mRNA and protein levels) in the vaginal wall (epithelium and stroma) in cervical cancer survivors treated with radiotherapy.

3 PARTICIPANTS AND METHODS

3.1 PARTICIPANTS (PAPERS I-III)

All studies were conducted at Radiumhemmet, Karolinska University Hospital and at the Department of Clinical Sciences at Danderyd Hospital, Stockholm. The studies were approved by the Regional Research Ethics Committee in Stockholm (Dnr 2003-753) and all the participants signed a written informed consent.

3.1.1 Cervical cancer survivors

Cervical cancer survivors treated with radiotherapy, between January 1, 2004, and December 31, 2007, were identified from the medical records at Radiumhemmet, Karolinska University Hospital. Inclusion criteria were diagnosis of cervical cancer treated with radiotherapy and age ≤51 years (the mean age for menopause for Swedish women). The time from the end of treatment to participation in the study was from two to five years. Exclusion criteria were recurrence of cervical cancer and significant comorbidity. In 2009 and 2010 letters explaining the objectives of the study were sent to the identified cervical cancer survivors inviting them to participate in the study (n=66). After one week, a research nurse telephoned the women. Of the total 66 cervical cancer survivors identified in the medical records, 34 women agreed to participate and were scheduled for a research visit (Figure 6).

3.1.2 Control women

As controls, 37 premenopausal women, aged ≤51 years undergoing benign gynecological surgery were recruited at Danderyd Hospital. Exclusion criteria for the controls were a history of cancer, current pregnancy, systemic disease, and vulvovaginal infections and/or inflammation. The most common reason for surgery among the control women was myoma. Conizations of the cervix uteri due to cervical dysplasia, laparoscopic surgery for sterilization, benign ovarian cysts or endometriosis were other causes for surgery.

Characteristics of cervical cancer survivors and control women are summarized in Table 4 and 5.

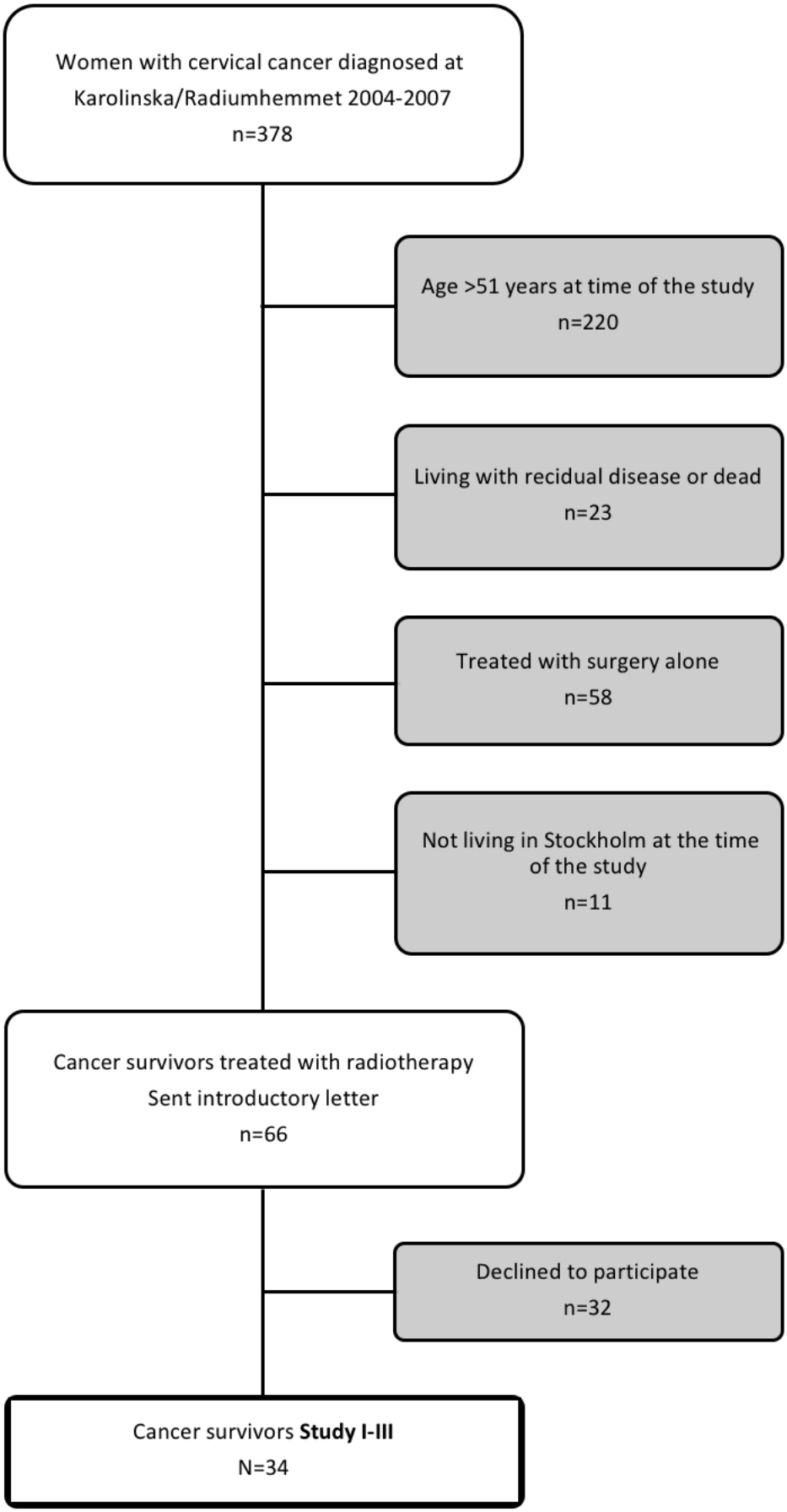


Figure 6. Flow-chart of the cervical cancer survivors in study I-III

Table 4. Cervical cancer survivor staging, histology and treatment.

FIGO stage – no. (%)	
IB1	16 (47)
IB2	4 (12)
IIA	6 (17)
IIB	5 (15)
IIIB	3 (9)
Histology – no. (%)	
Squamous cell carcinoma	25 (73)
Adenocarcinoma	4 (12)
Adenosquamous carcinoma	1 (3)
Glassy cell carcinoma	4 (12)
Treatment – no. (%)	
Preop brachytherapy + surgery	5 (15)
Preop brachytherapy + surgery + EBRT + chemo	8 (23)
Preop brachytherapy + surgery + EBRT	5 (15)
Surgery + EBRT + chemo (+ brachytherapy)	6 (18)
Surgery + EBRT (+ brachytherapy)	1 (3)
Primary radiochemotherapy (+ brachytherapy)	9 (26)

Abbreviations: EBRT, external beam radiotherapy; chemo, concomitant chemotherapy

Table 5. Characteristics of cervical cancer survivor and control women.

Clinical variables	Cervical cancer survivors N=34	Control women N=37
Age at time of study, mean (range)	41 (29-51)	40 (30-49)
Age – no. (%)		
<30	2 (6)	-
30-34	3 (9)	6 (16)
35-39	8 (24)	8 (22)
40-44	11 (32)	15 (40)
45-50	9 (26)	8 (22)
>50	1 (3)	-
Marital status – no. (%)		
Married or living with a partner	22 (65)	26 (70)
Has a partner but lives alone	1 (3)	3 (8)
Occasional partner	2 (6)	1 (3)
Single with no partner	9 (26)	6 (16)
Not stated	-	1 (3)
Menstrual cycle – no. (%)		
Regular	-	23 (62)
Irregular	-	11 (30)
Not stated	-	3 (8)
Ongoing oral contraceptive – no. (%)	-	14 (38)
Combined	-	3 (8)
Gestagen	-	11 (30)
Local hormonal therapy – no. (%)	13 (38)	-
Start at end of treatment	4 (12)	-
Start at first follow-up	1 (3)	-
Start when symptoms occurred	8 (23)	-
Systemic hormonal therapy – no. (%)	27 (79)	-
Estrogen	23 (67)	-
Combined	4 (12)	-
Start at end of treatment	19 (56)	-
Start at first follow-up	8 (34)	-
Start when symptoms occurred	3 (9)	-
Systemic and local hormonal therapy – no. (%)	9 (26)	-
Vaginal dilator – no. (%)	19 (56)	-
Started at end of treatment	10 (29)	-
Started at first follow up	6 (18)	-
Started when symptoms occurred	3 (9)	-

3.2 METHODS

3.2.1 Tissue sampling (papers I-III)

Vaginal biopsies were analyzed in study I-III. After a five-minute application of lidocaine/prilocaine cream (EMLA®, AstraZeneca, Sweden) two vaginal biopsies were taken from all the participants. The biopsies were taken with a 3 mm forceps at the three and nine o'clock positions, 3-4 cm proximal of the vaginal introitus. One biopsy was preserved in RNA*later* (Thermo Fisher Scientific Life Sciences, Waltham, MA) for further RNA isolation. The second biopsy was placed in phosphate buffered 4 % formaldehyde

solution for approximately 12 hrs. Paraffin embedding was performed according to a standard protocol. The samples were cut vertical to the surface, with a nominal thickness of 5 µm and mounted on slides. All biopsies and sections were handled and analyzed in a blinded manner throughout all analyses.

3.2.2 Clinical assessments (papers I-II)

One gynecological oncologist performed all the gynecological examinations in the cervical cancer survivors at a research visit. During the inspection, vaginal atrophy and the existence and quantification of telangiectasia were assessed. Atrophy was graded as absent (normal mucosa), mild (slightly pale mucosa, reduced folding), moderate (moderately pale, reduced folding) or severe (pronounced pallor, absent folding). Telangiectasia were scored as none (grade 0), single telangiectasia (grade 1), grouped telangiectasia (grade 2) and multiple telangiectasia (grade 3). Pelvic fibrosis and grade of vaginal inelasticity were determined by palpation as absent, mild (restricted to vagina), moderate (vagina and parametria) or severe (vagina and pelvis; "frozen pelvis"), Table 6. A vaginal measuring cylinder was used for measuring the vaginal length. One gynecologist examined all the control women according to protocol.

Table 6. Grading system for vaginal parameters.

Parameters/grading	**None=0**	**Mild=1**	**Moderate=2**	**Severe=3**
Atrophy				
Telangiectasia				
Pelvic fibrosis				

3.2.3 Radiation dose at biopsy site (papers I-III)

The cervical cancer survivors were treated before the use of 3D-guided BT. The EBRT plans were stored in an old planning system, from where the plans could not be retrieved. We therefore collected the prescribed field margins, target definition, set-up images, portal image films, treatment protocol and dose per fraction and the total prescribed dose for each patient. On portal image films the vaginal introitus was identified. The distance from the lower border of the EBRT field was measured and the EBRT dose at the biopsy site was calculated. For the BT we used orthogonal x-ray images and made an estimation of the delivered dose based on isodose-curves from the actual treatment for each patient. We calculated the EQD_2 doses for both the EBRT and the BT, obtaining the total dose at the biopsy site. In order to correlate radiotherapy-induced histological findings, the radiation dose at the biopsy site was calculated in two different treatment groups; (I) pre-operative BT, (II) BT + EBRT + surgery or primary radiochemotherapy.

3.2.4 Blood sampling and analyses (papers II-III)

Blood samples from the cervical cancer survivors were taken at the research visit. In the control women, blood samples were taken at the day of surgery. Serum estradiol, progesterone, follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone and sex hormone-binding globulin (SHBG) were determined. The serum levels of estradiol and testosterone were determined by radioimmunoassay (RIA), Spectria, Orion Diagnostica. The serum levels of progesterone, FSH, LH, SHBG were determined by chemiluminescent immunometric assay, Immulite, Siemens. Details of the hormone assays are presented in Table 7. Free testosterone was determined by a formula using total testosterone, SHBG and a fixed concentration of albumin (40g/L) [198].

Table 7. Hormone assays used in study II-III

Hormone	Method	Reference value	Intraassay precision (CV)	Interassay precision (CV)
Estradiol	RIA	Premenopausal: 105-1399 pmol/L Postmenopausal: 11-50 pmol/L	2.8-18.1 %	5.8-17.6 %
Progesterone	ChLIA/CLIA	Follicular phase: ND-3.6 pmol/L Luteal phase: 3.0-68 pmol/L Midluteal phase: 19-76 pmol/L Postmeopausal: ND-3.2 pmol/L Oral contraceptives: 1.1-2.9 pmol/L	6.3-16 %	5.8-16 %
FSH	ChLIA/CLIA	Premenopausal: 1.2-21 IU/L Postmenopausal: 21.7-153 IU/L	2.6-3.7 %	5.8-6.7 %
LH	ChLIA/CLIA	Premenopausal: ND-77 IU/L Postmenopausal: 11.3-39.8 IU/L	4.8-6.5 %	7.2-26 %
Testosterone	RIA	ND-3.3 nmol/L	3.1-8.9 %	5.2-11.6 %
SHBG	ChLIA/CLIA	18-114 nmol/L	4.1-7.7 %	5.8-12 %

Abbreviations: CV, coefficient of variation; RIA, radioimmunoassay; ChLIA/CLIA, chemi-luminescent immunometric assay; FSH, follicle-stimulating hormone; LH, luteinizing hormone; ND, not detectable; SHBG, sex hormone-binding globulin

3.2.5 Measuring elastic fibers (paper I)

The elastic fibers in the connective tissue were visualized by their autofluorescent properties in unstained sections in three images from each patient and control. The specimens were placed in the microscope (Axioplan 2, Zeiss) under a 40× objective and three random-sampled images per section were captured, with a 3-chip CCD RGB-color camera (Sony DXC-9100P). The images were digitized and stored in 8-bit RGB-mode in the computer under the control of MicroGOP 2000s (Context Vision) image-analysis software. The image size was equal in all specimens. Finally, the image-analysis program

calculated the area fraction of the elastic fibers (in percent) in each slide by dividing the total elastic fiber area by the total tissue area of the three images [199].

3.2.6 Measuring collagen content (paper I)

To analyze the collagen content in the tissue, sections were stained with Sirius red, which binds covalently to the collagen molecule and is thus highly specific. Thereafter, the density of the collagen fibers was measured by intensitometry, i.e. calculation of the intensity of light passing through the collagen in the sections using the same equipment as above, but programmed for intensitometric measurements. The specimens were placed on a light table and the light was calibrated with a filter set of known densities. Optical density was estimated by detecting and measuring the area fraction for pixels in the specimen within certain defined grey level intervals, ranging from black = 0, to white = 255 (8-bit grey level). Subsequently, the total intensity, obtained from each grey level interval, was compared with the total area of the section [200, 201]. The collagen fibers were divided into four different panels (A-D) according to their optical densities; panel A representing the fibers with the highest density and panel D the fibers with the lowest density. For each participant three sections were evaluated and the result is presented as median percentage of total pixel area.

3.2.7 Epithelial measurements (paper II)

For capturing the complex structure of the vaginal epithelium with the dermal papilla, a computer-assisted method analyzed the epithelial structures [12]. The epithelial thickness, the number and the form of the dermal papillae, as well as and the dermal papillae distance were measured in µm. The following epithelial parameters were analyzed; the distance from dermal papilla top to epithelial surface (DPS), interdermal papilla distance (DPD), the dermal papilla width (DPW), the distance from basal layer to the epithelial surface (BLS) and the number of dermal papillae (Figure 7). In the microscope (Axioplan 2, Carl Zeiss, Jena, Germany) the specimens were placed under a 5x-objective and the specimen's image was captured in 8-bit RGB-mode into the computer (Sun SparcStation 20, Sun Microsystems Computer Corp., CA) equipped with Micro-GOP 2000s (Context Vision, Linköping, Sweden) image analysis software. The threshold on the basal layer was set manually, thereafter the epithelial profile was outlined and the measurement procedure begun with the described parameters [12]. Each sample was analyzed from the left side to the right with five equally separated intervals of 411 µm each.

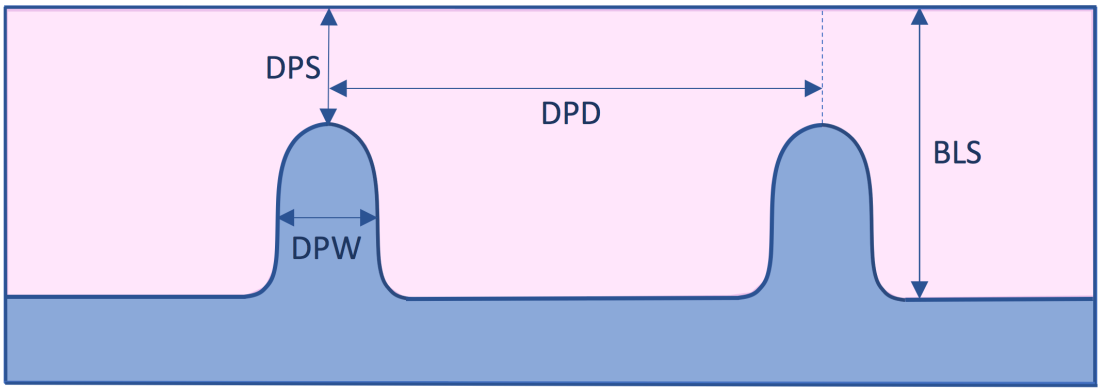


Figure 7. Epithelial parameters measured in each sample
Abbreviations: DPS, distance from dermal papilla top to epithelial surface; DPD, interdermal papilla distance; DPW, dermal papilla width; BLS, distance from basal layer to epithelial surface.

3.2.8 Blood vessels (paper II)

To analyze the presence of blood vessels, sections were immunohistochemically stained with antibodies against coagulation factor VIII von Willebrand, present in endothelial cells [202]. The samples were placed in the microscope (Axioplan 2, Carl Zeiss, Jena, Germany) under the 20× objective and images were captured and digitally stored. Three grey scale pictures were made and then further enhanced with a stretch filtering and two contextual image procedures. The resulting picture was thresholded, presenting an accurate image of the vessels. Subsequently, a stereological cycloid grid was put on the image and the intercept points, where the grid touching the vessel wall, were identified (Figure 8). The program calculated the intercept points; six intercept measurements on each biopsy and the morphometrical analyses of blood vessels were made, by measuring the vessel surface area per unit tissue volume (mm^2/mm^3) [203, 204].

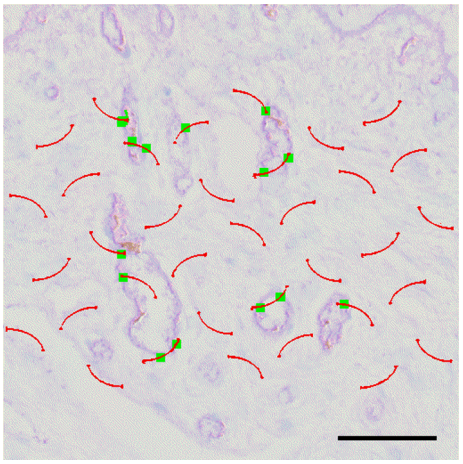


Figure 8. Vaginal wall specimen stained with antibodies against coagulation factor VIII, with red cycloid measurement grid and green identifications marks at the vessel walls. Bar = 100 µm.

3.2.9 Nerve fibers (paper II)

The specimens were also stained with antibodies against protein gene product 9.5 (PGP 9.5), for the detection of nerve fibers (Figure 9) [6]. The volume fraction of IHC stained nerves was estimated in percent of the volume of examined tissue [201].

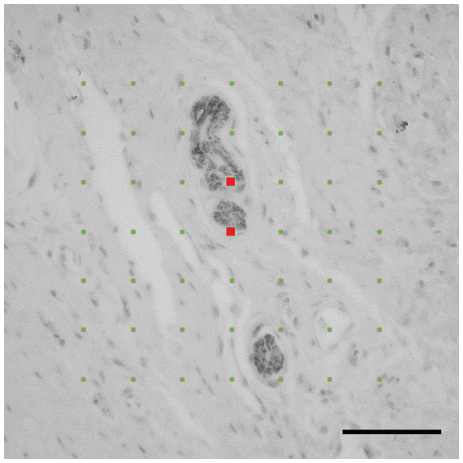


Figure 9. A specimen from the vaginal wall staid with antibodies against protein gene product 9.5 for detecting nerve fibers, with a grid showing red identifications marks at the nerve fibers. Bar = 100 μm.

3.2.10 Questionnaire (paper II)

The questionnaire was developed from a study-specific questionnaire, described and used by Bergmark *et al*, 1999 [143]. The original questionnaire was based on in-depth interviews with cervical cancer survivors, and face-to-face validated with cervical cancer survivors and women without a history of cancer treatment. In our questionnaire, we included 49 questions concerning demographic data, information on quality of life, psychological health and assessment of sexual dysfunction. Thirty-nine questions were specifically related to sexuality, such as sexual interest, vaginal lubrication and genital swelling, vaginal length reduction and inelasticity, superficial and deep dyspareunia and frequency of intercourse and orgasm.

For questions on quality of life and psychological health a seven-point Visual Digital scale was used. Study persons were asked to estimate their wellbeing the last six months and responses on the scale ranging from 1-5 were considered as low to moderate and 6-7 as high. For responses on levels of anxiety and depression, 1-2 were considered absent or low and 3-7 moderate to high [205].

The specific questions on sexuality were covering overall sexual function, intercourse function and specific physical sexual symptoms like lubrication, vaginal elasticity and reduction in vaginal length. The answers were given in four to seven verbal categories, or that the symptom was not relevant.

For example;
In some women, the elasticity of the vagina could feel reduced during sexual intercourse.

Have you noticed that symptom during the last 6 months?
☐ *Not relevant* - I have not engaged in sexual intercourse (or similar activity) during the last 6 months
☐ No, not at all
☐ Yes, a little reduced elasticity
☐ Yes, moderately reduced elasticity
☐ Yes, very reduced elasticity

3.2.11 RNA preparation and reverse transcription (paper III)

Total RNA from vaginal biopsies were purified with RNeasy® Mini Kit (Qiagen GmbH, Hilden, Germany) according the procedure for fibrous tissue recommended by the manufacturer. Two μg of total RNA from each sample was reverse transcribed at 37°C for 60 min in a final volume of 40 μL.

3.2.12 Real-time PCR analysis (paper III)

For mRNA analyses of ERα, ERβ, G-protein-coupled ER-1 (GPER), PRA, PRB, AR, connective tissue growth factor (CTGF) and RPLP0 (ribosomal protein P0; a housekeeping gene) real-time PCR was performed in an iCycler™ iQ Real Time PCR System (Bio-Rad Laboratories, Inc, CA USA). The cDNAs, corresponding to 50–100 ng RNA, were added to 12.5 μL of iQ™ SYBR® Green Supermix (Bio-Rad) and 0.3 μM of each oligonucleotide primer in a final volume of 25 μL PCR reactions.

All reactions were performed in duplicates. A melting curve analysis was performed in all experiments to confirm the purity of the PCR products. Details on the used oligonucleotide primers are presented in Supplemental Table 1, paper III. All primers, except PRB, were designed to span an intron/exon boundary or to be on different exons, to eliminate amplification of contaminating DNA. A negative control containing a RNA sample without reverse transcription was included in each PCR assay. The mRNAs for PRA and PRB are transcribed from same gene, PRB mRNA being longer than PRA mRNA, why it is not possible to design primers that will detect only PRA mRNA. The PRAB primers will detect both PRA and PRB mRNAs as they are directed to the common sequence of the mRNAs while the primer pair for PRB mRNA will detect a part of the PRB mRNA that is unique and not translated into PRA.

To standardize the quantification method, RPLP0 was selected out of several tested housekeeping genes as an invariable internal control and used to standardize the quantification method. The PCR amplification rate and the cycle threshold (Ct) values were related to a standard curve using iCycler iQ Optical System Software (Bio-Rad). The values of relative expression of the genes of interest were normalized against the RPLP0 product.

3.2.13 Immunohistochemistry (paper III)

Tissue sections were deparaffinized in xylene, then rehydrated in graded ethanol and microwaved for antigen retrieval in sodium citrate buffer for 10 min. Non-specific endogenous peroxidase activity was blocked by 3 % hydrogen peroxide in methanol for 10 min at room temperature after a wash in buffer. Sections were exposed to normal horse or swine serum for 30 min. Thereafter the tissue sections were incubated with the primary antibody. Negative controls were obtained by replacing the primary antibody with the specie relevant IgG. The bound enzyme was visualized by the application of 3,3´-diaminobenzidine after the incubation with horseradish peroxidase-avidin biotin complex. Counterstaining with haematoxylin and dehydration was performed before mounted with Pertex®. Details on dilutions, buffers and incubation times for the different antibodies are shown in Supplemental Table 2, paper III.

3.2.14 Image analysis (paper III)

To quantitatively assess nuclear immunostaining, a Leica microscope was connected to a computer using Colorvision software (Leica Imaging System Ltd. Cambridge UK). Ten fields were selected at random from the squamous epithelium and the stroma, for quantification of the area of positively immunostained (brown) nuclei. Analyzes were done separately for the two tissue types. When it was not possible to obtain ten separate fields due to lack of representative tissue, all epithelium and/or stroma was measured in those tissue samples. The total area of positively stained nuclei was determined and expressed as a ratio of the total area of cell nuclei (brown reaction product + blue haematoxylin) by the Colorvision software. This method was used for analyzing the nuclear receptors ERα, ERβ, PRA, PRB and AR.

3.2.15 Manual scoring (paper III)

GPER and CTGF immunostaining was measured by manual scoring due to their mainly cytoplasmic staining. Two independent observers scored, blinded for the identity of the samples, on a four-point scale from negative (0), (+) faint, (++) moderate and (+++) strong immunostaining. This method shows good consistency between two independent observers [206].

3.3 STATISTICS

Data was analyzed using IBM SPSS Statistics 22.0 and SAS statistical software package. The analysis of data from the survivors and controls were performed using Student's *t*-test or Mann-Whitney U-test wherever appropriate. Fisher's exact test was used for comparing categorical variables between patients and controls. Analysis was performed by ANOVA on ranks (Kruskal-Wallis test) for comparing differences in more than two treatment groups and significances were evaluated by Dunn's test all compared to all.

Pearson correlation coefficient R was used to test correlation between hormonal levels and epithelial measurements variables. Spearman's rank correlation test was used for

correlating vaginal atrophy and the responses of the questionnaires to the epithelial measurements.

The responses to the questionnaire were dichotomized, and the results were presented as relative risks (95 % confidence intervals), calculated as a proportion of the cancer survivors reported a particular problem divided by the proportion of the controls.

The significance level was set at $p<0.05$.

4 RESULTS

4.1 CLINICAL FINDINGS (I-III)

The cervical cancer survivors had a shorter vagina, median 7.0 cm (range 5.0-9.0), compared to the control women, median 10.3 (7.0-13.0), $p < 0.001$. Clinical findings of atrophy, telangiectasia and pelvic fibrosis were commonly observed in different degrees in the cancer survivors (Table 8).

Table 8. Clinical findings of cervical cancer survivor and control women.

	Cervical cancer survivors N=34	**Control women** N=37
Length of vagina (cm), median (min-max)	7.0*(5.0-9.0)	10.3 (7.0-13.0)
Length of vagina (cm) – no.		
<6	3/34	0
6-7.9	22/34	1/37
8.0-9.9	9/34	9/37
>10	0	27/37
Vaginal atrophy – no. (%)	31 (91)	0
No signs	3 (9)	37 (100)
Mild	15 (44)	-
Moderate	15 (44)	-
Severe	1 (3)	-
Telangiectasia – no. (%)	28 (82)	0
None	6 (18)	37 (100)
Mild	16 (47)	-
Moderate	11 (32)	-
Severe	1 (3)	-
Pelvic fibrosis – no. (%)	33 (97)	0 (0)
No signs	1 (3)	37 (100)
Mild	16 (47)	-
Moderate	14 (41)	-
Severe	3 (9)	-

*$p<0.001$

4.2 RADIATION DOSE AT BIOPSY SITE (I-III)

Treatment with preoperative BT and radical surgery showed minimal dose at biopsy site, median 0.0 Gy (range 0.0-9.0). Five out of the 34 patients were cured with this treatment modality. Patients that received additional EBRT postoperatively and patients treated with primary radiochemotherapy received high dose at biopsy site; median 44.3 Gy (38.8-49.3) for postoperative EBRT and 53.4 Gy (47.9-69.0) for primary radiochemotherapy.

In the further analyses the cervical cancer survivors were divided into two treatments groups. Group I were women treated with preoperative BT and surgery (n=5), receiving minimal radiation dose at the biopsy site, and group II were women that had received EBRT and high radiation dose at biopsy site (n=29).

4.3 HORMONAL ANALYSIS (II-III)

Serum estradiol was corresponding on a group level in cervical cancer survivors and control women. The median serum estradiol was 140.8 pmol/L (IQR 83.7-203.8) in the cervical cancer survivors, compared to 189.4 pmol/L (72.9-337.0), ns, in controls. The cervical cancer survivors in the two treatments groups, with minimal and high radiation dose at biopsy site (group I and II), also had corresponding levels of serum estradiol. Serum estradiol was median 108 pmol/L (IQR 59.0-147.0) in the women with minimal dose at biopsy site (n=5), compared to 145 pmol/L (85.0-208), ns, in the patients with high radiation dose at biopsy site (n=29).

As expected, hormonal treatment with estradiol affected the serum level of estradiol. Cervical cancer survivors treated with both systemic and local estradiol (n=9) had significantly higher level of serum estradiol, median 185.2 pmol/L (IQR 123.0-257.2), compared to women with only local estradiol (n=4), median 25.3 pmol/L (8.0-100.3) or no hormonal therapy (n=3), 20.7 pmol/L (17.4-24.0), p=0.002. Cervical cancer survivors treated with only systemic estradiol (n=18) had median 144.1 pmol/L (106.2-208.6), no significant difference compared to the other hormonal treatments.

Serum levels of estradiol in the control women were affected by the use of contraceptives. In control women using contraceptives (n=14), the serum estradiol was as anticipated low with median 80.4 pmol/L (IQR 33.6-206.1), compared to 218.6 pmol/L (83.2-455.4) in controls in the follicular phase (n=14) and 283.2 pmol/L (33.6-206.1) in controls in the luteal phase (n=8), p=0.022.

Due to their history of cancer treatment, serum progesterone level was significantly lower and FSH and LH levels were higher in the cervical cancer survivors, compared to the control women.

The SHBG level was higher in the cervical cancer survivors, median 77.7 nmol/L (IQR 50.5-108.0), compared to 49.5 nmol/L (34.0-71.0) in the control women, p=0.003, but no significant differences were found between various groups concerning hormonal treatment. No significant differences were found in serum levels of testosterone.

4.4 HISTOLOGICAL FINDINGS

4.4.1 Elastin (I)

In the cervical cancer survivors, the area fraction of elastin in the vaginal wall was greater, 10.0 % (range 0.8-18.1), compared to the control women 3.4 % (0.9-11.6), $p<0.001$. There was a marked difference in the organization and distribution of the elastic fibers (Figure 10). In the control women, the elastin fibers were situated in a thin layer under the basal membrane of the epithelium. In the cervical cancer survivors, the elastin fibers were thickened and were located throughout the connective tissue forming a disorganized pattern.

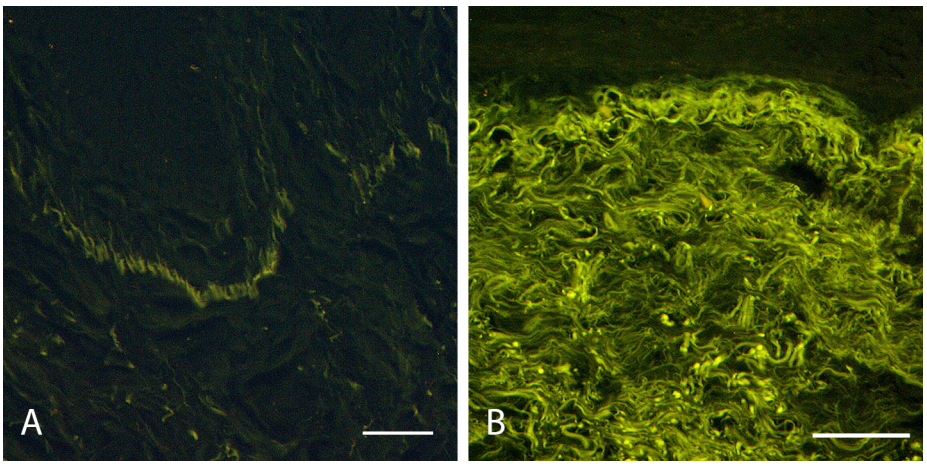


Figure 10. Autofluorescent elastin fibers in a sub-epithelial layer in the vaginal wall in control women (A), and thick elastin fibers in the connective tissue in cervical cancer survivors (B). Bar = 200 µm.

4.4.2 Collagen (I)

Collagen fibers were found in all the samples from the vaginal wall, but the distribution of fibers with different densities varied between the cervical cancer survivors and the control women. The collagen with the highest density (panel A) was most common in the cervical cancer survivors, median 43.7 % (range 1.1-84.2), compared to the controls, median 24.6 % (2.3-68.2), $p<0.001$. In the control women collagen fibers with the different densities were rather equally distributed. Cervical cancer survivors treated with EBRT (high radiation dose at biopsy site) had more collagen with the highest density, median 53.9 % (range 8.7-84.2), compared to the cancer survivors with minimal radiation dose at biopsy site 27.0 % (1.1-36.4), $p<0.01$.

4.4.3 Epithelial measurements (II)

In the computer-assisted epithelial measurements the cervical cancer survivors had a thinner epithelium with a shorter distance from the basal layer to the epithelial surface (BLS), median 339 µm (IQ range 220-419), compared to 382 µm (IQ range 317-464) in the control women, $p<0.05$. As a further sign of a reduced epithelial volume, the distance

between the dermal papillae (DPD) was longer in the biopsies from the cervical cancer survivors, median 381 µm (IQ range 293-1002) compared to 279 µm (IQ range 239-337) in the controls, $p<0.001$. Consequently, the number of the normal papillae was lower in the specimen from the cervical cancer survivors, median 3.3 (IQ range 1.0-4.3), as compared to controls, 4.0 (IQ range 3.0-4.8), $p<0.05$. No differences in the distance from dermal papilla top to epithelial surface (DPS) or dermal papilla width (DPW) between survivors and control women were found (Figure 11).

There were no differences in any of the epithelial measurements between patients with minimal radiation dose at the biopsy site (preoperatively BT only), compared to those who had received a high radiation dose (EBRT + BT).

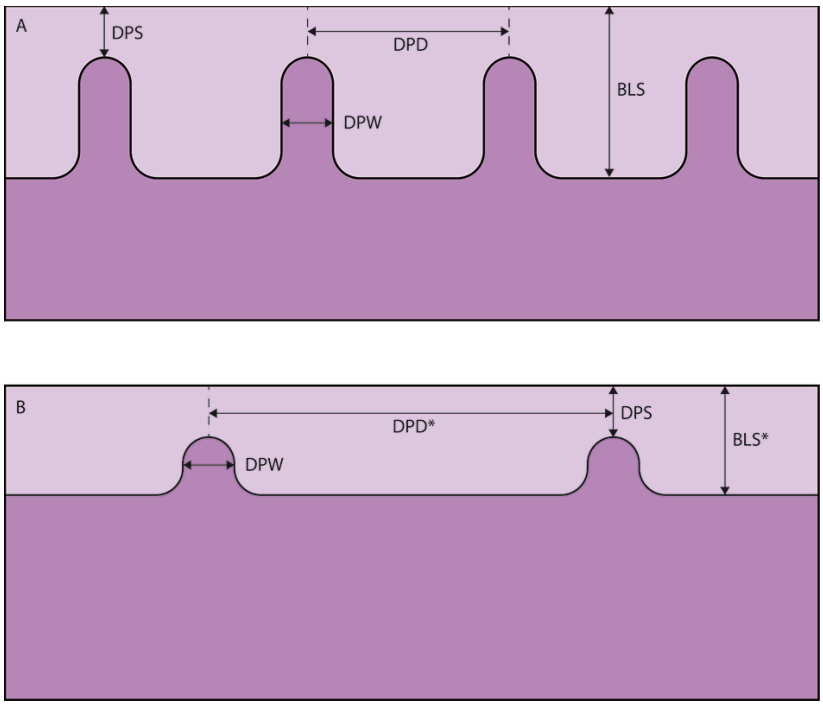


Figure 11. An illustration of the vaginal wall with the epithelial measurements in control women (A), and cervical cancer survivors with thinner epithelium (shorter BLS) and a longer distance between the dermal papilla (DPD) (B). *$p<0.05$
Abbreviations: DPS, distance from dermal papilla top to epithelial surface; DPD, interdermal papilla distance; DPW, dermal papilla width; BLS, distance from basal layer to epithelial surface.

4.4.4 Blood vessels (II)

There was no difference in the mean surface area fraction of blood vessels per tissue volume in the vaginal wall in the cervical cancer survivors, compared to the control women. In the cancer survivors, the mean area fraction of blood vessels per tissue volume was 106.2 mm^2/mm^3 (SD 26.6), compared to the controls 101.6 mm^2/mm^3 (SD 25.6), ns.

4.4.5 Nerve fibers (II)

There was a low volume fraction of nerves in the specimen from the vaginal wall from both cervical cancer survivors and control women, with no significant differences. The nerve tissue had a maximum of 1 % of the tissue volume in 75 % of the specimens; in the other 25 % there were a maximum of 4 % nerve tissue.

4.5 QUESTIONNAIRE (II)

The cervical cancer survivors reported more vaginal sexual symptoms, compared to the control women. Among the cancer survivors, 39 %, compared to 3 % among the control women reported moderate or substantially insufficient vaginal lubrication for sex, relative risk (RR) 12.6, 95 % confidence interval (CI) 1.7-91.3. Accordingly, 36 % of the cancer survivors reported regular use of lubricants, compared to 3 % in the controls, RR 10.3 (95 % CI 1.4-75.6). Almost half of the cancer survivors (45 %) reported moderate or substantially reduced elasticity of vagina, compared to 7 % of the controls, RR 6.5 (95 % CI 1.6-26.3). Also, the cancer survivors more often reported reduced genital swelling when sexually aroused and reduction in vaginal length during intercourse. No significant differences were seen in superficial and deep dyspareunia and the frequency of vaginal intercourse was similar in cancer survivors and control women.

4.6 PCR (III)

In the cervical cancer survivors (n=26), the relative mRNA expression of ERα in the vaginal wall was lower, median 0.5 (IQR 0.6-1.0), compared to the control women (n=29), median 0.8 (0.6-1.0), p=0.007. No differences in the relative mRNA expression of ERα were found between cervical cancer survivors treated with minimal radiation dose at biopsy site compared to the cancer survivors treated with high radiation dose at the biopsy site. Higher relative mRNA expression of CTGF was found in the cervical cancer survivors, median 0.6 (IQR 0.2-1.4), compared to controls median 0.2 (0.2-0.3), p=0.002.

4.7 IMMUNOHISTOCHEMISTRY (III)

In the vaginal biopsies, the ERα positive cells were mostly distributed along the basal membrane of the epithelium in both cervical cancer survivors and control women, and less frequently seen in the stroma. There was a lower immunostaining of ERα in the vaginal epithelium of the cervical cancer survivors (n=34), median 58.9 % (IQR 42.1-72.8), compared to controls (n=37), median 73.9 % (64.2-79.5), p=0.001. No significant differences were seen in the stroma (Figure 12). Further, when comparing the cervical cancer survivors that had received minimal or high radiation dose at biopsy site, there was a lower immunostaining of ERα in cancer survivors with high radiation dose (n=29), median 56.8 % (37.3-69.9), compared to cancer survivors with minimal radiation dose at biopsy site (n=5), 75.2 % (61.7-84.0), p=0.046. The latter group showed values similar to the control women. Also, the amount of ERα positive cells in the stroma was lower in cancer survivors with high radiation dose, compared to cancer survivors with minimal radiation

dose at biopsy site. No significant differences were found concerning immunostaining of ERβ and GPER.

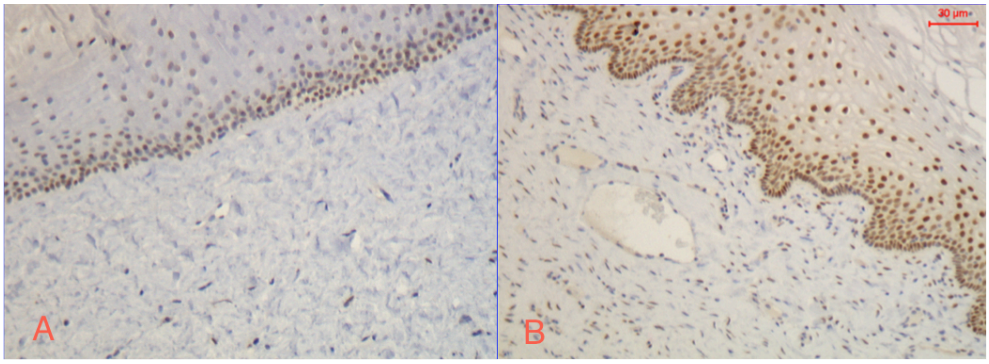


Figure 12. Images of immunostaining of ERα in the vaginal wall in women treated for cervical cancer (A) and control women (B). Bar = 30 µm.

The AR expressing cells were found both in the stroma and the epithelium. The cervical cancer survivors had a lower expression of AR in the epithelium, median 32.3 % (IQR17.4-51.6), compared to 68.6 % (61.4-80.3) in the control women, $p<0.001$, but no difference in the stroma. Thereafter, when comparing the cancer survivors according to radiation dose at biopsy site, we found a lower immunostaining of AR in cancer survivors with high radiation dose, median 31.1 % (13.8-46.8), compared to cancer survivors with minimal radiation dose at biopsy site, 56.4 % (44.2-63.5), $p=0.01$. Also, the amount of AR positive cells in the stroma was lower in cancer survivors with high radiation dose, compared to cancer survivors with minimal radiation dose at biopsy site.

5 DISCUSSION

5.1 METHODOLOGICAL CONSIDERATIONS

Validity refers to the level of which the tests in a study are measuring what it is supposed to measure and the reliability the consistency of the measurement. Our clinical studies are limited according to the small sample size, but we aimed for good internal validity by minimizing systemic errors through a proper study design.

Selection bias

Selection bias can occur when the study population's likelihood of participating in the study leads to a different result than we would have received if the whole target population were enrolled. Of the 66 cervical cancer survivors meeting the inclusion criteria and receiving a letter explaining the study, only 34 agreed to participate. There were no significant differences according to age, tumor stage and treatment modalities between participants and non-participants. The remaining 32 women most commonly referred to psychological reasons for not participating or the inconvenience of an extra visit at the clinic. The women who chose to participate could theoretically have more sexual symptoms than non-participants, and therefore be more interested in the study. On the other hand, non-participants could be women with more symptoms and discomfort during gynecological examination, thus denying participation. This could have biased our results regarding vaginal changes and sexual functioning towards worse or better.

The control women were all scheduled for benign gynecological surgery. The reason for surgery varied from diagnosis of myoma, cervical dysplasia and endometriosis, to procedures of non-pathological origin, such as changing of IUD and sterilization. It is reasonable to believe that these control women had more sexual symptoms than a cross section of age-matched women from the general population, biasing the results concerning sexual function towards worse. So, the differences in sexual function could have been even larger if the premenopausal control women were selected randomly from the general population. We have no basis to believe that the vaginal histological changes could be biased by the fact that these women needed benign gynecological surgery.

Information bias

Information bias is caused if the data, collected in the study, is incorrect. Information bias is divided into differential and non-differential misclassification. Differential misclassification, non-random, could have occurred in study II when the cervical cancer survivors answered the questionnaire on sexual dysfunction. The cancer survivors were informed of the study objectives in advance. This fact could have made these women more aware of general symptoms and symptoms of sexual dysfunction, compared to the control women, who received the information of the study the same day as they participated and filled out the questionnaire.

Non-differential misclassification is caused by random measurement errors, which could have occurred in the hormonal and histological analyses. Random errors could weaken the outcome, causing null results even though an association is present. The hormonal analyses were performed in batches for minimizing random errors caused by assay variation. Another possibly source of random errors could have been the fact that the blood tests were taken at different time of the day and in the control women regardless of menstrual cycle. This could have diluted the results and made a wider distribution of values, but the positive associations found are therefore not believed to be caused by non-differential misclassification.

The clinical findings and grading of vaginal atrophy, pelvic fibrosis and telangiectasia were performed by one gynecological oncologist in all cervical cancer survivors. A grading scale were established and evaluated by two observers before the start of the study. Vaginal length was measured by a vaginal measuring cylinder in the same manner in all study women. The vaginal biopsies in the cancer survivors were taken after a five-minute application of lidocaine/prilocaine cream with a 3 mm forceps. Punch biopsies would have been preferred for optimal histological analysis, but was not possible to use due to the vaginal biopsy sites. The vagina in the cancer survivors is typically narrowed and with reduced rugae, which complicates the taking of biopsies. In the control women, the biopsies were easier to take and the risk for mechanical damage of the biopsies were less. Some control women were under general anesthesia when the biopsy was taken. Even so, lidocaine/prilocaine cream was used in the same way, to minimize the risk of differences between groups due to the effect of the cream.

The cervical cancer survivors in the studies were treated before modern radiotherapy modality with IMRT and 3D-guided BT was introduced. The radiation-dose at biopsy site was estimated by the information from the planning system and X-ray images by the same observers in each patient.

Concerning the histological analysis for elastin and collagen the methods were chosen based on the knowledge and experience in the research group. The methods are simple and robust and based on proven stereological concepts. Elastin is a large and long molecule that is very difficult to solve and can therefore not be chemically quantified. For this reason, histological morphometric methods are much better suited. The fact that the elastin molecule is large causes it to autofluoresce. Unstained sections could therefore be used, reducing the risk of staining artifacts. Since we do not have a well-defined reference volume, the amount of elastin was specified as an area fraction, not a volume fraction. Three different images from each sample were then analyzed for a more reliable result.

Collagen fibers exist in a large amount in the study biopsies. In fact, most of the connective tissue consists of collagen. For this reason, we used intensitometry to calculate the light passing through the sections, which not only investigate the amount of collagen but also the density of the collagen fibers. Sirius red was used to stain the collagen, known for being specific and stable [207]. Three different sections were analyzed from each study participant.

In studies analyzing the morphology of the vaginal epithelium, different methods have been used. Most frequently the epithelial thickness has been analyzed as the length from the epithelial surface to the basal membrane. For a more accurate epithelial measurement a computerized, stereological method was used in study II. This method not only defines the epithelial thickness, but also measures the epithelial structures with details of the dermal papillae.

We studied the presence of blood vessel and nerve fibers in the biopsies by using IHC. When analyzing blood vessels, we used a method measuring the surface area fraction of blood vessels per tissue volume. In a histological section the same blood vessel could be seen in different parts due to its winding appearance. By measuring the area fraction instead of just counting the vessels in the sections, we achieved a more accurate measurement of the presence of blood vessels. One limitation is that the biopsies were small and thereby only showing the epithelium and the superficial part of the connective tissue. Important unidentified changes in blood vessels and nerve fibers could be present in the deeper part of the vaginal wall.

The steroid hormone expression was analyzed at both mRNA and protein levels, using real-time PCR and IHC. By using both methods the findings are strengthen. In the PCR analyses, adequate quantity and quality of RNA could only be extracted in 26 out of 34 of the samples from the cancer survivors, compared to 29 out of the 37 samples from the control women. A similar loss was obtained in both groups resulting in a reduction of total sample size. This could have limited the possibility to find small differences between the study groups. IHC for validation of the steroid hormone expression at the protein level is a clinically commonly used method. The limitations are the risk of subjectivity assessing the slides and the risk that differences in staining weaken the results. To overcome this, the samples were handed in a blinded manner and two independent observers performed the manual scoring. In the smallest samples, when ten randomly selected fields from the epithelium and stroma were not possible to obtain, all epithelium and/or stroma were analyzed.

Confounding

A confounding factor is a variable that is an independent risk factor for the outcome and is associated with the exposure, but not an effect of the exposure. A confounding factor can both overrate and underrate a result. A potential confounding factor in the hormonal analyses and histological measurements of the vagina is age. Therefore, when the study design was set, we chose to limit the study population to women age ≤51 years. In study II and III, the hormonal levels and hormonal treatment could be potential confounding factors and we therefore collected these data. To adjust for hormonal levels correlation tests were performed. The heterogeneous material concerning hormonal treatments in the cancer survivors and menstrual cycle and contraceptives in the control women could have influence the results. This must be taken into account in the interpretation of the findings.

Smoking is a potential confounding factor in the studies. We have no information on smoking habits from the control women, but in the cervical cancer survivors 11 out of 34

were smokers at the time of the study. Smoking habits could affect the radiotherapy-induced fibrosis, but a larger study group would have been needed for adjusted data analysis.

Confounding from comorbidity was controlled by restriction, as being one of the exclusion criteria in both cancer survivors and control women.

5.2 MAIN FINDINGS AND IMPLICATIONS

5.2.1 Clinical findings

In the cervical cancer survivors, we found clinical signs of atrophy, pelvic fibrosis and telangiectasia, which was in contrast to the absence of these signs in the control women. The vaginal length was reduced in the cancer survivors, compared to control women. The cancer survivors that had received external pelvic radiation showed the most prominent changes in atrophy, pelvic fibrosis and vaginal length. These findings are in concordance with the observations on vaginal changes reported by Kirchheiner *et al*. [183].

5.2.2 Histological findings in the connective tissue

In the vaginal connective tissue, we found fibrosis and high elastin content *(*elastosis) in cervical cancer survivors treated with radiotherapy. The most prominent fibrotic changes were found in the cancer survivors with high radiation dose at biopsy site, i.e. the women that had been treated with external pelvic radiation. To our surprise, we found a higher area fraction of elastin in the cancer survivors, compared to controls. However, when we studied the microscopic slides we found that the distribution and characteristics of the elastin fibers were changed. In the control women, the elastin fibers formed a thin sub-epithelial layer along the basal membrane, but in the cancer survivors, compact elastin fibers were spread throughout the connective tissue. These results together with the clinical finding of inelastic vaginal walls in the cancer survivors indicate that the elastin is dysfunctional. In the correlations analysis, we found no significant difference in elastin content in the cancer survivors with high radiation dose at biopsy site, compared to survivors that only received minimal radiation dose. This result might be due to the small sample size, with only five patients in the latter group. High prevalence of elastin in the connective tissue is seen in the connective tissue disease of scleroderma, which cause thicken, harder skin, with loss of elasticity [208]. The histological findings also have morphological similarities to actinic elastosis seen in sun-damaged skin [209].

The collagen showed higher density in the cancer survivors compared to controls. The collagen with the highest density, i.e. fibrosis, were also more common in the cancer survivors that had received a high radiation dose at biopsy site, compared to the patients treated with preoperative BT and surgery and thereby only received minimal radiation dose at the distal part of the vagina. Consequently, we conclude that the fibrosis is radiotherapy-induced. These connective tissue changes can alter the function of the vaginal wall and play an important role in a morphological explanation of the clinical findings and physical symptoms in cervical cancer survivors.

For a functional vaginal wall the blood vessels and nerve fibers are essential. We found no differences in the area fraction of superficial blood vessels and nerve fibers between cancer survivors and controls. In our biopsies only the upper part of the vaginal wall was represented, therefore possible deeper located changes important for the microcirculation of the tissue could not be studied. A clinical observation when taking the biopsies were that there was almost no bleeding in the cancer survivors, compared to the control women, where in a few cases even a stitch was needed for hemostasis. Photoplethysmography could be a better method for analyzing the vessel function in the vaginal wall [210].

5.2.3 Histological findings in the epithelium

We found morphological changes in the cervical cancer survivors with reduced vaginal epithelial volume, compared to control women. The cancer survivors had a shorter distance from the basal layer to the epithelial surface and sparse dermal papillae, indicating not only a thinner epithelium but also a reduced epithelial volume and function. These morphological findings correlate to our clinical findings of atrophy. The dermal papillae contain blood vessels and are essential for the function of the avascular epithelium. A reduced number of dermal papillae can negatively influence the nourishment by the capillaries and further reduce the growth of the mucosa. To obtain effect of systemic estrogen, the hormone has to reach the receptors of the target cells. As expected, we found a positive correlation between serum levels of estradiol and the epithelial thickness when including all study participates. The cancer survivors were all treated with radiotherapy resulting in complete cessation of the endogen ovarian estrogen production. A majority of the cancer survivors were using systemic hormonal treatment and on a group level there was no difference in the serum estradiol, implying that the survivors were sufficiently substituted. Even so, vaginal atrophy was found both clinically and morphologically, supporting previous findings that radiotherapy affects the responsiveness to estrogen [186]. One may speculate if the reason for this could be the fibrotic changes in the connective tissue and the loss of dermal papillae with impact on the microcirculation and/or changes in the expression of hormone receptors.

5.2.4 Hormonal levels

The analyses of the serum hormonal levels showed postmenopausal values for FSH, LH and progesterone in the cervical cancer survivors, compared to the premenopausal control women. There was no difference in serum estradiol on a group level, between the cancer survivors and the control women. However, the material was heterogeneous concerning the hormone therapy, both regarding systemic and local estradiol treatment. This reflects the variation in clinical recommendations and compliance after the cervical cancer treatment is completed [211]. The estradiol levels varied, depending on the hormonal therapy, with the highest levels in the cancer survivors treated with both systemic and local estradiol, and the lowest levels in those with only local estradiol or no treatment at all. On the other hand, the control women with contraceptives showed as expected lower levels of estradiol compared to the cycling controls. In the cancer survivors, 79 % used systemic estradiol and a corresponding number of controls (62 %) were not using contraceptives, which explain the equal estradiol level between cancer survivors and controls.

5.2.5 Sex steroid hormone receptors

In the cervical cancer survivors, we found a lower expression of ERα in the vaginal wall at both mRNA and protein levels, compared to control women. The cancer survivors that had received external radiation as part of their treatment, with a high radiation dose at the vaginal biopsy site, showed lower expression of ERα in both epithelium and stroma, compared to the survivors treated with BT and surgery. To our knowledge, there are no previous studies on the steroid receptor expression in the vaginal wall after treatment of cervical cancer with radiotherapy. However, the dynamic interplay between the steroid hormone receptors and the serum hormone levels has been demonstrated in several studies in pre- and postmenopausal women with vaginal prolapse. A lower ERα expression in the vaginal wall has been found in postmenopausal women, compared to premenopausal women [114, 115], with increased expression in women treated with local estrogen [116], but less with systemic administration [122]. Our findings with lower ERα expression in a patient group were the majority were using hormonal therapy, might indicate that the vaginal mucosa is less likely to respond to estrogen therapy after radiation. At the same time, we must not forget that this finding applies to patients two to five years after the end of the cancer treatment, when the long-term side effects are considered permanent. Early start and accurate dosage of local estrogen therapy might preserve the mucosal responsiveness to estrogen.

We also found a lower AR expression in the epithelium in the cancer survivors compared to control women. In the cancer survivors with high radiation dose at the biopsy site, the immunostaining of AR was lower in both epithelium and stroma, compared to survivors with minimal radiation dose. AR expression in the vaginal wall in women with no history of cancer, have shown higher expression in premenopausal, compared to postmenopausal women [121]. Even though the knowledge on the effects of AR and testosterone on the vagina is limited, studies on rabbits have shown AR involvement in the vaginal hemodynamics and vaginal mucification [212]. Changes in the AR expression could therefore have effects on the vaginal sexual response.

CTGF is a central mediator of tissue remodeling and fibrosis [213]. Our finding of higher mRNA expression of CTGF in the vaginal wall in the cancer survivors, compared to controls, is in concordance with our previous results of radiotherapy-induced fibrosis in the connective tissue. Studies on mice have indicating that inhibition of CTGF can prevent and reverse the process of radio-induced pulmonary fibrosis [214].

5.2.6 Sexual function

Sexual dysfunction in cervical cancer survivors is complex and has multifactorial etiology. Persistent changes in sexual function in cervical cancer survivors are reported and result in considerable distress [143]. We found that the cancer survivors reported more physical symptoms like reduced lubrication and elasticity, reduction in vaginal length and sexual response with genital swelling, compared to the control women. These symptoms could be mediated by the morphological changes in the vaginal wall. The reduction in length and

elasticity of the vagina is probably partly due to the radiotherapy-induced fibrosis, but also by the surgery in the patients where radical hysterectomy was part of the treatment [215].

Lubrication and genital swelling during sexual arousal is dependent on increased blood flow, but the decreased epithelial volume and the changed connective tissue in the cancer survivors could also affect the ability to respond sexually. Dyspareunia is reported in several previous studies as one of the most distressing symptoms after cervical cancer treatment with radiotherapy [162]. We found no difference regarding dyspareunia between the cancer survivors and the control women. This might be due to the small sample size, but we must also take into consideration that the women in the control group were planned for gynecological surgery. The differences in sexual function could have been even larger if the control women were randomly selected from the general population.

6 GENERAL CONCLUSIONS

- In cervical cancer survivors treated with radiotherapy, the connective tissue in the vaginal wall is changed, with development of fibrosis and elastosis. The highest risk of vaginal fibrosis is seen in women treated with external radiation.

- Cervical cancer survivors treated with radiotherapy have a reduced vaginal epithelium volume and clinical signs of vaginal atrophy, compared to control women with corresponding serum levels of estradiol.

- Irradiated cervical cancer survivors have an increased risk of vaginal sexual symtoms; such as insufficient lubrication, inelasticity, reduced genital swelling when sexually aroused and reduced vaginal length.

- Cervical cancer survivors treated with external radiation have reduced ERα and AR expression in the vaginal mucosa. The vaginal atrophy after cervical cancer treatment could partly be due to decreases in sex steroid hormone receptor expression.

7 FUTURE PERSPECTIVES

Cervical cancer survivors are a rising number of women at risk for chronic side effects. The improvements in treatment result in increased survival rates, but the advanced treatment must also induce minimal morbidity for reaching our goal to cure with the best possible quality of life. Sexual dysfunction is reported as one of the most distressful symptoms after the end of treatment, which point out the need for efforts to cure with minimal vaginal symptoms and maintained sexual health. Our studies serve as a step towards better knowledge of the morphological changes in the vaginal wall in cervical cancer survivors.

Studies are required to evaluate therapy developments in radiotherapy and new surgical techniques in the treatment of cervical cancer. In the progress of modern radiotherapy, we need to gain more knowledge of the distribution of the vaginal radiation dose and thereby improving the individually treatment planning, minimizing the vaginal side effects. The relationship between vaginal dose and the risk for vaginal side effects needs to be evaluated in future studies.

An intervention study is needed to evaluate the potential effect of local estrogen therapy in preventing vaginal morbidity and sexual dysfunction. The timing, dosage and administration form of the treatment ought to be studied in a large patient group with long-term follow-ups. The optimal start of treatment is probably immediately upon completion of radiotherapy. Usually, the prescribed dosage of the topical estrogen is the same as for treating vaginal atrophy in postmenopausal women with no history of radiation. Higher doses and more frequent administration might be needed to reduce vaginal symptoms after radiation. The various applications of topical treatment, cream, gel or vaginal tablets, could also have different effects that needs to be further evaluated for more individualized guidance.

The use of vaginal dilators is widely recommended in guidelines, but still the evidence is weak concerning long-term benefits [216]. The use of dilators during the radiotherapy is not supported. It has been suggested to start dilation once the acute inflammation has settled, but it is still unclear whether early use of dilators can prevent vaginal stenosis. It should be possible to set up a study protocol for a randomized controlled trial investigating optimal start, frequency and long-term effect. However, there are several issues to consider. In previous studies, it has been shown that women developing stenosis are more reluctant to continue with the dilators and for some women the use of dilators is stressful [217].

The benefit of other topical drugs, such as benzydamine (NSAID) and products containing A and E vitamins, as well as surgical reconstruction of the vagina, has been reported in minor studies [218-220], but further evaluation is needed.

The heterogeneity in the cancer survivors in this thesis regarding hormonal treatment and use of vaginal dilator, demonstrate the need to strengthen the rehabilitation programs and strive towards a multi-professional organization. Information to the patients before, during and after the treatment can be further developed to improve the compliance. Current

clinical guidelines address the issue and give recommendations in the rehabilitation phase, but health-care providers must be organized and prepared to meet the requirements.

Sexual symptoms can be difficult to address both for patients and professionals. Written information and clinically designed questionnaires can be helpful and give the patients a more standardized follow-up regarding sexual health. Studies on side effect prevention and information regarding sexual issues in women treated for gynecological cancer, may further improve the cancer survivors' quality of life.

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